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Since *Burkholderia mallei* is of significance as an agent of bioterrorism (Category B, Centers for Disease Control, U.S.) and biological warfare, the development of effective vaccines and treatments is of particular concern. Our understanding of the disease caused by this organism is rapidly emerging, and we must move forward with our studies on the pathogenesis of this disease in order to develop new and effective vaccines and/or therapies against this organism. There is considerable dual use potential, since the development of vaccines and treatments can provide important items to assist the World Health Organization, to assist in providing a state of preparedness in case of bioterrorism attacks and to assist signatories of the Biological and Toxin Weapons Convention under Article X of the Convention. The long-term objective of our research is to define at a molecular level the pathogenesis of disease due to *Burkholderia ssp.* My laboratory is recognized internationally as a world leader in the understanding of the pathogenesis of disease caused by *Burkholderia ssp.*, and considerable advances in understanding of the diseases caused by these organisms have already been made by my laboratory.

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INTRODUCTION

Glanders, caused by *Burkholderia mallei*, is a significant disease for humans due to the serious nature of the infection. It is recognized that *B. mallei* is an organism with tremendous infectivity that poses a significant hazard to humans exposed to aerosols containing this organism. Our knowledge of the pathogenesis of disease due to *B. mallei* is lacking. At present, no effective vaccines are available against this organism, and information on the treatment of this organism with antibiotic therapy is also not available.

The basic studies that we are performing on the pathogenesis of disease due to *B. mallei* are acutely needed, and the information gained from these studies will provide a knowledge base that is required to rationally design new modes of therapy directed against this organism. The long-term objective of our research is to define at a molecular level the pathogenesis of disease due to *B. mallei* and to develop immunoprotective vaccines against these organisms for use in humans.

Since glanders is of military significance as a biological warfare agent, the development of an effective vaccine and treatments are of particular concern. Our understanding of the disease caused by *B. mallei* is minimal, and we must move forward with these studies in order to develop new and effective vaccines and/or therapies against this organism. There is considerable dual use potential, since this disease is important in various areas of the world. Development of vaccines and treatments can, therefore, provide important items to assist the World Health Organization and to assist signatories of the Biological and Toxin Weapons Convention under Article X of the Convention.

BODY

Burkholderia pseudomallei and *Burkholderia mallei* are gram-negative bacilli that are the causative agents of melioidosis and glanders, respectively. Glanders is primarily a disease of solipeds, but incidental infections may also occur in humans (1),(2). Manifestations of glanders may generally be grouped as acute suppurative infection, acute pulmonary infection, acute septicemic infection and chronic suppurative infection. The incubation period of this disease may range from 1-14 days, depending on the route of infection (3). The prognosis of the localized or chronic forms is favorable; however, the acute septicemic form is usually fatal (3). Melioidosis is described as a glanders-like infection that may affect humans and a variety of animals. Melioidosis may be recognized as inapparent infection, asymptomatic pulmonary infiltration, acute localized suppurative infection, acute pulmonary infection, acute septicemic infection or chronic suppurative infection (3). The incubation period for melioidosis may range from 2 days to 29 years (3),(4). The use of antimicrobials has improved the prognosis for melioidosis; however, even with intensive antibiotic therapy, the mortality rate for the septicemic form is still 40% (5). Infections caused by both *B. pseudomallei* and *B. mallei* are often due to either direct inoculation into wounds and skin abrasions or to inhalation (3),(6),(7),(8). Although these diseases bear a striking resemblance both clinically and pathologically, they are epidemiologically quite different. While *B. pseudomallei* is an environmental saprophyte isolated from soil and water capable of causing disease, *B. mallei* is considered to be an obligate parasite of horses, mules, and donkeys, with no other known natural reservoir (2),(3). The genomes of *B. pseudomallei* and *B. mallei* are highly homologous; however *B. mallei* is considered to be a more refined pathogen due to the smaller size of its chromosome (9).

Melioidosis is recognized as an important health problem in Southeast Asia and Northern Australia, and it poses a concern due to increased travel and military involvement in endemic regions. Isolated cases of melioidosis have been identified in a number of other regions as well, including China, the Indian sub-continent, sub-Saharan Africa, France, Mexico, Brazil, and the Caribbean (7),(10),(11). Although glanders has been eradicated from North America and Western Europe, the disease still persists in South America, Eastern Europe, Africa and Asia (3). In addition, both *B. pseudomallei* and *B. mallei* are classified as category B biological agents, and both have been utilized previously as agents for biological warfare (12). Due to the importance of these diseases and the fact that the causative agents are not well characterized on a molecular level, there has been an increased interest in the identification and characterization of virulence determinants in these organisms in order to develop therapeutic and preventative strategies for the treatment of melioidosis and glanders.

We have written a number of reviews on melioidosis and glanders. These manuscripts are attached as appendices:

Appendix #1 - Reckseidler-Zenteno, S.L., Ling, J., Moore, R.A., and Woods, D.E. 2003. Recent Research Advances on the Pathogenesis of Melioidosis and Glanders. *Recent Res. Devel. Infect. Immun.* 1:187-202.

Appendix #2 - Brett, P.J., and Woods, D.E. 2000. Pathogenesis of and Immunity to Melioidosis. *Acta Trop.* 74:201-210.

Appendix #3 - Woods, D.E., DeShazer, D., Moore, R.A., Brett, P.J., Burtnick, M.N., Reckseidler, S.L., and Senkiw, M.D. 1999. Current Studies on the Pathogenesis of Melioidosis. *Microbes and Infection* 2:157-162, 1999.

Appendix #4 - Burtnick, M.N., Moore, R.A., Tribuddharat, C., and Woods, D.E. 2000.

Antibiotic Resistance Mechanisms of *Burkholderia pseudomallei*. Res. Adv. Antimicrob. Ag. and Chemother. 1:1-6.

GENETIC TOOLS FOR THE STUDY OF MELIOIDOSIS AND GLANDERS

Much of our increased understanding of melioidosis and glanders has resulted from the rapid increase in studies utilizing molecular biological techniques. As a result of these efforts a variety of molecular tools and methods have been found to be of great use with both *B. pseudomallei* and *mallei*. These tools and techniques are described below.

Signature tagged mutagenesis

Signature tagged mutagenesis (STM) has been used successfully to identify virulence factors in a number of bacterial pathogens. This technique essentially involves infecting an animal with a pool of transposon mutants each containing a unique oligonucleotide tag. Bacteria are then recovered from the animal and the recovered tags compared to the original bank of tags used to infect the animal. Tags that are missing represent mutants which were unable to survive in the host and likely defective in a gene contributing to virulence. In a report by Atkins *et al* (13), signature tagged mutants were subjected to *in vivo* screening in Porton Outbred (PO) mice. A non-capsulated mutant of *B. pseudomallei* was identified which was attenuated in virulence when infection occurred via i.p. infection but was still virulent when challenged via an i.v. route. The acapsular mutant was found to contain an interruption in the mannosyltransferase gene which is required for synthesis of capsular polysaccharide. Interestingly, the gene affected was

one of the same capsule biosynthesis genes previously shown as essential for virulence in a hamster model (14). Importantly, this group has demonstrated a means of successfully using STM in *B. pseudomallei*. Although there are no reports yet which describe use of STM in *B. mallei*, it is likely that this procedure could be modified for use in this pathogen as well.

Promoter library and microarray analysis

Expression profiling of pathogenic bacteria allows for the identification of bacterial genes that are differentially regulated in the host. Within this class of genes are those that adapt the organism to host-specific microenvironments or encode virulence determinants. As recovery of organisms from infected host cells are low and contaminated with host genetic material, current attempts to study *B. pseudomallei* virulence gene expression are done *in vitro*. This is based on the assumption that the expression of genes encoding virulence determinants can often be induced *in vitro* by simple modifications of laboratory media (15).

Reporter gene fusions are often used in the discovery and characterization of *cis*-acting promoter regions and *trans*-acting regulatory proteins. In bacterial systems, reporters such as *lacZ* (β -galactosidase), *phoA* (alkaline phosphatase), *cam* (chloramphenicol transacetylase) and *lux* (bacterial luciferase) have been used. Of these systems, the *lux* reporter gene is advantageous as a sensitive, real-time measure of gene expression without disrupting the cell. In addition to identifying and characterizing promoter regions and regulatory proteins, the *lux* reporter system has been used as a marker for monitoring intracellular bacteria within human cells (16) or bacterial virulence gene expression during infection of tissue cell lines or animals (17). The reporter system can also be used in luminescence assays in *in vitro* conditions.

In our laboratory, we are presently attempting to use the *lux* reporter system to identify promoter and regulatory systems that are involved in the expression of virulence factors. These virulence factors will be investigated further for their pertinence to the pathogenesis of melioidosis caused by *B. pseudomallei*. Briefly, a random promoter library is constructed in *B. pseudomallei* 1026b. The library is then screened for light production when grown under differing growth conditions *in vitro*, that is in conditions that mimic host-specific microenvironments. Our preliminary findings suggest that *B. pseudomallei* is able to respond to extracellular signals such as differing concentrations of iron, magnesium, manganese and glucose, as well as osmotic stress.

In a complimentary approach, we have placed oligonucleotides of a set of known virulence determinants on microarray slides. *B. pseudomallei* 1026b are grown in differing growth condition, such as varying concentrations of iron, glucose and salt, and the RNA isolated to provide quantitative expression data *in vitro*. A similar attempt is underway for microarray expression profiling of known virulence determinants for *B. mallei*.

Subtractive hybridization

Subtractive hybridization has been used successfully to identify an important virulence determinant in *B. pseudomallei* (14). Subtractive hybridization experiments between *B. pseudomallei* and *B. thailandensis* were performed using a PCR-Select Bacterial Genome Subtraction Kit (Clontech) as described by the manufacturer except that hybridization temperature was increased from 63°C to 73°C to account for the high G+C content found in these two species. In these experiments DNA from *B. thailandensis* was used as driver DNA, and DNA from *B. pseudomallei* was used as tester DNA. The PCR products obtained from this

procedure were cloned into pZero-2.1 (Invitrogen) and pPCR (18) and enriched for *B. pseudomallei* sequences. The *B. pseudomallei*-specific sequences were then sequenced and the sequences analyzed for homology using the BLASTX program.

Using this approach one clone was identified which had weak homology with WbpX, a glycosyltransferase involved in LPS biosynthesis from *P. aeruginosa*. This gene was insertionally inactivated in wild type *B. pseudomallei* 1026b to generate *B. pseudomallei* SR1015. SR1015 was shown to be capsule deficient and avirulent in a hamster infection model and thereby identified the capsule as an important virulence determinant in *B. pseudomallei*.

Subtractive hybridization has also been used to identify transposable genetic elements in *B. pseudomallei* and in *B. thailandensis* (19). The transposable elements identified in *B. thailandensis* represent the first transposons identified in that species.

Transposon Mutagenesis

The use of transposon mutagenesis has proven to an extremely effective means of identifying potential virulence determinants in *B. pseudomallei*. DeShazer *et. al.* (20), first described the use of the transposon *Tn5*-OT182 to isolate motility mutants and to characterize the flagellin structural gene in *B. pseudomallei*. This transposon contains a promoterless β -galactosidase gene which allows for the formation of β -galactosidase transcriptional fusions provided the transposon has inserted downstream of a promoter in the correct orientation. The transposon also contains a *ColE1* origin of replication which allows rapid self-cloning of flanking DNA regions (21) and contains *bla* and *tetAR* genes for selection via ampicillin and/or tetracycline. In *B. pseudomallei* tetracycline at 50 ug/ml is used for selection. *Tn5*-OT182 was

successfully used in 6 out of ten *B. pseudomallei* strains tested with up to a tenfold difference in efficiency between strains

Two *Tn5*-based transposons containing truncated *phoA* genes have been used successfully in *B. pseudomallei*, *B. mallei* and *B. thailandensis*. Thus, virulence determinants which are located extracytoplasmically can be identified (22),(23) Use of a *phoA* based transposon required that acid phosphatase negative mutants be constructed (24) using allelic exchange with the gentamycin sensitive strains *B. pseudomallei* DD503 (25) and *B. thailandensis* DW503 (24). *TnPhoA* was delivered to these strains using the vector pRT733; however, the efficiency in *B. mallei* was much less than that found in *B. pseudomallei*. Cloning of flanking DNA was accomplished by digesting transposon containing chromosomal DNA and cloning it into the vector pBR322. Efficiency of this step was low, likely because of the size the transposon (approx. 5kb) and because the cloning vector pBR322 has an effective insert limit of about 7kb. Thus, flanking DNA sizes greater than 2 kb would result in low efficiency at this cloning step. Construction of another alkaline phosphatase-based transposon, mini-*OphoA* (26) circumvented these problems by incorporating an origin of replication in the transposon thus allowing for self-cloning of flanking DNA regions. As well, transposition of mini-*OphoA* was more efficient than *TnPhoA* in *B. mallei*. Both of these systems have been used successfully in *Burkholderia* ssp. for the identification of genes encoding exported proteins.

Atkins *et.al.* (27), utilized a transposon mutagenesis approach to generate a *B. pseudomallei* mutant that was interrupted in the *ilvI* gene which encodes an enzyme essential for branched chain amino acid synthesis. The mutant generated by this approach was significantly attenuated in a mouse model and provided protection against infection by the wild type parent. Unlike the systems described above which rely on conjugation to introduce the transposon into recipient

strains, transposon mutants in this study were generated by electroporation of plasmid pUTminiTn5Km2 (28) into *B. pseudomallei* strain 576. Flanking DNA of transposon mutants were sequenced using a PCR protocol (29) which amplified DNA fragments adjacent to the transposon.

Useful vectors for genetic studies of *B. pseudomallei* and *B. mallei*

Allelic exchange allows for the introduction of defined mutations into the chromosome and is a useful method for determining gene function. Allelic exchange involves two successive homologous recombination events between a wild type gene and a cloned gene containing either a deletion or an antibiotic cassette and often employs vectors which contain the conditionally counterselectable marker *rpsL*. This gene confers a streptomycin sensitive phenotype in strains which are streptomycin resistance by virtue of a *rpsL* mutation. Allelic exchange using the *rpsL*-based vector pKAS46 (30) was used to construct *B. pseudomallei* DD503 in which a deletion was introduced into the *amr* locus of *B. pseudomallei* RM101 (25), thus rendering the strain susceptible to aminoglycoside antibiotics and suitable for additional studies involving allelic exchange.

Use of pKAS46 with strain DD503 has allowed introduction of defined mutations in the form of deletions and antibiotic resistance cassettes into a variety of genes involving virulence including the flagellin structural gene (*fliC*) (20), serum resistance genes (31) and polymyxin resistance genes (32). *B. pseudomallei* DD503 also allows use of a variety of vectors which employ aminoglycoside resistance as a selective marker which otherwise could not have been used due to the inherent aminoglycoside resistance found in wild type *B. pseudomallei*.

Vectors known to be useful for complementation of *B. pseudomallei* genes *in trans* include pBBR1Tp (33), pUCP28T, pUCP29T and pUCP31T (34). These vectors allow blue/white selection, contain multiple cloning sites and use trimethoprim or gentamicin resistance as selectable markers. Recently, pBHR1, a mobilizable broad-host-range vector, has been shown to be useful for complementation studies in *B. mallei* (35). Vectors useful for insertional inactivation in *B. pseudomallei* include pSKM11 (36) and pKNOCK-Cm (37) (38). A summary of useful transposons and vectors is shown in Table 1.

Table 1

Transposons	Description	Reference
pOT182	PSUP102(Gm)::Tn5-OT182 Cm ^r Gm ^r Ap ^r Tc ^r	(21)
pUTminiTn5 Km2	Km ^r	(28)
pRT733	oriR6K mobRP4 TnphoA;Ap ^r Km ^r	(39)
pmini-OphoA	pMB1 oriR, Tn5 tnp, RP4 oriT, phoA; Gm ^r	(26)
Vectors		
pKAS46	Mobilizable allelic exchange vector; \square -Dependent R6K replicon; Ap ^r rpsL ⁺ (Sm ^s)	(30)
pKNOCK-Cm	Mobilizable, Broad host range suicide vector Cmr	(37)
pSKM11	Mobilizable allelic exchange vector ; IncP mob;ColE1 ori;Ap Tc	(36)
p34E-Tp	Cassette cloning vector; Tp ^r	(33)
pBBR1Tp	Mobilizable broad-host-range vector; Tp ^r	(33)
pUCP28T	Broad host range vector:OriT pRO1600 ori; Tp ^r	(34)

pUCP29T	Broad host range vector: OriT pRO1600 ori; T ^r	(34)
pUCP31T	Broad host range vector: OriT pRO1600 ori; Gm ^r	(34)
pBHR1	Mobilizable broad host range vector; Km ^r Cm ^r MoBiTec	(35)

We have published a number of manuscripts on the development and use of genetic tools to study the pathogenesis of disease due to *B. pseudomallei* and *B. mallei*. These are attached as appendices:

Appendix #5 - Deshazer, D., and Woods, D.E. 1999. Pathogenesis of Melioidosis: Use of Tn5-OT182 to Study the Molecular Basis of *Burkholderia pseudomallei* Virulence. J. Infect. Dis. Antimicrob. Ag. 16:91-96.

Appendix #6 - Deshazer, D., Brett, P.J., Burtnick, M.N., and Woods, D.E. 1999. Molecular Characterization of Genetic Loci Required for Secretion of Exoproducts in *Burkholderia pseudomallei*. J. Bacteriol. 181:4661-4664.

Appendix #7 - Bolton, A.J., and Woods, D.E. 2000. A Self-Cloning Minitransposon *phoA* Gene-Fusion System Promotes the Rapid Genetic Analysis of Secreted Proteins in Gram-Negative Bacteria. Biotechniques. 29(3):472-474.

Appendix #8 - Reckseidler, S.L., DeShazer, D., Sokol, P.A., and Woods, D.E. 2001. Detection of Bacterial Virulence Genes by Subtractive Hybridization: Identification of the Capsular Polysaccharide of *Burkholderia pseudomallei* as a Major Virulence Determinant. Infect. Immun. 69:34-44.

Appendix #9 - DeShazer, D., Waag, D.M., Fritz, D.L., and Woods, D.E. 2001. Identification of a *Burkholderia mallei* Polysaccharide Gene Cluster by Subtractive Hybridization and Demonstration That the Encoded Capsule is an Essential Virulence Determinant. Microb. Pathogen. 30:253-69.

Appendix #10 - Woods, D.E., Jeddloh, J.A., Fritz, D.F., and DeShazer, D. 2002.

Burkholderia thailandensis E125 Harbors a Temperate Bacteriophage Specific for *Burkholderia mallei*. J. Bacteriol. 184:4003-4017.

Appendix #11 - Tribuddharat, C., Moore, R.A., Baker, P., and Woods, D.E. 2003.

Characterization of Class A β -Lactamase Mutations of *Burkholderia pseudomallei* That Confer Selective Resistance Against Ceftazidime or Clavulanic Acid Inhibition. Antimicrob. Ag. Chemother. 47:2082-2087.

Appendix # 12 - Burtnick, M.N., Brett, P.J. and Woods, D.E. Identification of Genes Encoding Secreted Proteins Using Mini-*OphoA* Mutagenesis. In: Methods in Molecular Microbiology, Vol. 205, *E. coli* Gene Expression Profiles. P.E. Vaillancourt, Ed. pp. 329-338. 2002.

Appendix # 13 - Inglis, T.J.J., Robertson, T., Woods, D.E., Dutton, N., and Chang, B.J. 2003. Flagella-Mediated Adhesion by *Burkholderia pseudomallei* Precedes Invasion of *Acanthamoeba*. Infect. Immun. 71:2280-2282.

INFECTION MODELS FOR THE STUDY OF MELIOIDOSIS AND GLANDERS

A number of animal models have been developed in order to understand the pathogenesis of melioidosis and glanders and to function as systems for assessing the efficacy of antibiotics and vaccine candidates *in vivo*. Also the role of the host in infection can be suggested.

One of the most frequently used animal models for the study of *B. pseudomallei* pathogenesis is the Syrian golden hamster model of acute *B. pseudomallei* infection. It was demonstrated many years prior that a number of animals could be infected with *B. pseudomallei* and the hamster proved to be the most susceptible animal (40). Since Syrian golden hamsters are exquisitely sensitive to infection by *B. pseudomallei*, a hamster model of acute melioidosis was

developed by Brett *et al.* to assess the lethality of a number of *B. pseudomallei* strains (41) (42). Female Syrian golden hamsters, 6-8 weeks of age, were inoculated intraperitoneally (i.p.) with 100 μ l of logarithmic phase cultures adjusted to the appropriate dilution in phosphate-buffered saline. The animals were then monitored for morbidity and mortality, and LD₅₀ values were calculated. When the hamsters were inoculated with those strains predicted to be highly virulent based on secretion profiles, 5/5 of the hamsters in each group succumbed to the infection within 48 hours, while those strains predicted to be less virulent, had no effect on the hamsters. The LD₅₀ values for the highly virulent strains were determined to be <10 cfu, while the LD₅₀ values for the weakly virulent strains were 10⁶ cfu, representing a >10⁵-fold difference in virulence. Bacterial counts from the blood of the animals inoculated with the highly virulent strains displayed a marked bacteremia, while bacteria could not be detected in the blood of the animals inoculated with the lowly virulent strains. The lethal effects produced by the virulent strains were found to be consistent with the clinical manifestations observed in patients with acute septicemic melioidosis (41),(42). This study provided the basis for the classification of non-pathogenic isolates of *B. pseudomallei* as a novel species, *B. thailandensis* (43).

Further studies employing the Syrian hamster model of infection have contributed to the identification of genetic factors responsible for the enhanced virulence of *B. pseudomallei* relative to *B. thailandensis*. A system combining subtractive hybridization between *B. pseudomallei* and *B. thailandensis*, insertional mutagenesis of genes unique to *B. pseudomallei*, and animal virulence studies using the Syrian hamster model was developed by our laboratory in order to identify *B. pseudomallei* virulence determinants (14). As described below, this methodology led to the identification of a capsular polysaccharide as an important virulence determinant in *B. pseudomallei* pathogenesis (14).

The Syrian hamster model of acute melioidosis has been utilized in a number of studies to assess the role of *B. pseudomallei* virulence factors in disease. A number of mutant strains generated by our laboratory have been tested for virulence using this model (14) (20),(25),(31),(42),(44). For example, a number of *B. pseudomallei* Tn5-OT182 mutants susceptible to the bactericidal action of 30% normal human serum and lacking the O-polysaccharide (O-PS) of LPS were found to be less virulent in the Syrian hamster compared to wild type, establishing the importance of O-PS in serum resistance and virulence (31). In contrast, there was no difference in the virulence of a Tn5-OT182 flagellin mutant and type II secretion mutants in this model compared to wild type *B. pseudomallei*, suggesting that these are likely not significant virulence determinants in this animal model (20),(42).

The Syrian hamster model has also been used for investigating the efficacy of antimicrobial agents for the treatment of melioidosis. Subcutaneous injections of sulfadiazine, streptomycin and penicillin were used to treat hamsters infected i.p. with $1-6 \times 10^3$ *B. pseudomallei* (40). Sulfadiazine treatment was effective in preventing death, while streptomycin and penicillin were not. *B. pseudomallei* is relatively susceptible to sulfadiazine but is resistant to streptomycin and penicillin *in vitro* (45) (25). Therefore, there is a correlation between the activity of these antimicrobial agents *in vitro* and *in vivo* using this model (42). There have been no reported studies recently on the activity of antibiotics using the Syrian hamster model of infection; however, this model would be useful for the evaluation of new antibiotics for the treatment of melioidosis.

Rats have been shown to be relatively resistant to *B. pseudomallei* infection; however, the LD₅₀ of *B. pseudomallei* in infant rats can be lowered significantly by i.p. injection of the diabetogenic compound streptozotocin (STZ) (46),(42). Infant rats weighing 30-40 g were

inoculated i.p. with 80 mg of STZ/kg of body weight for 2 consecutive days to induce diabetes. One week after the final injection of STZ, the infant rats were infected with *B. pseudomallei*. The LD₅₀ of *B. pseudomallei* for nondiabetic infant rats was determined to be $>1 \times 10^8$ bacteria, while the LD₅₀ in STZ-induced diabetic infant rats was approximately 1×10^4 bacteria (46). The infection of diabetic infant rats with *B. pseudomallei* resulted in acute septicemic melioidosis and death within 7 days. This animal model was developed because a significant percentage of septicemic melioidosis patients are diabetic, and this model enabled the authors to investigate the effect of insulin on *B. pseudomallei* infection (see 46). This animal model has been successfully utilized in passive immunoprophylaxis studies to demonstrate the protective capacity of antisera raised to purified *B. pseudomallei* O-PS, flagellin protein, and an O-PS-flagellin conjugate, which are potential vaccine candidates for the prevention of melioidosis (47),(48),(49). The infant diabetic rat model has also been useful in assessing the relative virulence of *B. pseudomallei* transposon mutants (20),(31),(44).

As mentioned above, melioidosis may present as an acute pneumonia, an acute septicemia, a chronic infection involving long lasting suppurative abscesses or a subclinical infection that may remain undetected for many years until activated by a traumatic event or a decrease in immunocompetence (50). Recently a number of mouse models have been developed in order to represent the various manifestations of melioidosis and the major routes of infection by *B. pseudomallei*. Previous studies demonstrated that BALB/c mice were highly susceptible to infection with virulent *B. pseudomallei*, while C57Bl/6 mice were found to be relatively resistant (51). Two mouse models of experimental *B. pseudomallei* infection were developed using these inbred strains (52). BALB/c and C57Bl/6 mice were inoculated i.v. in the lateral tail vein with a number of serial dilutions of *B. pseudomallei*, and after 10 days, the LD₅₀ values were calculated.

The LD₅₀ values for the BALB/c and C57Bl/6 mice were determined to be 4 cfu and 2.5×10^4 cfu, respectively. The course of infection in BALB/c mice was similar to acute melioidosis, while infection of C57Bl/6 mice was found to mimic chronic melioidosis. Infection of BALB/c mice was rapidly fatal, resulting in bacteremia and death by 96 hours. In contrast, C57Bl/6 mice did not suffer from bacteremia and remained asymptomatic for up to 6 weeks. However, both strains of mice were found to contain high bacterial loads in the liver and spleen. Infection by *B. pseudomallei* in BALB/c and C57Bl/6 mice was further characterized to provide a basis for investigations on the pathogenesis of melioidosis (53). Similar to the previous studies, these authors observed marked organotropism of *B. pseudomallei* for the spleen and liver in both strains of mice, with the highest bacterial load in the spleen. Electron micrographs of the spleen demonstrated intracellular replication of *B. pseudomallei* within membrane-bound phagosomes. In addition, the development of abscesses and granulomatous lesions consistent with those seen in human melioidosis occurred in both the spleen and liver of both mouse strains and was more prevalent in the BALB/c mice (53). Also BALB/c mice developed a pronounced splenomegaly compared to C57Bl/6 mice, which is observed in acute systemic melioidosis of humans (53). The mechanism for the increased resistance of C57Bl/6 mice to infection of *B. pseudomallei* remains unclear; however, it is thought that the difference may be due to an enhanced innate immune response, possibly effective non-specific bactericidal activity of C57Bl/6 macrophages, a more pronounced Th1-type immune response in the C57Bl/6 mice and an inability of BALB/c mice to contain the infection at sites of inflammation (52),(53),(54).

The BALB/c mouse model of acute disseminated *B. pseudomallei* infection was utilized to study the efficacy of cefpirome, a fourth generation cephalosporin and potentially effective agent for the treatment of melioidosis (55). The results of this study demonstrated that

ceftazadime and cefpirome are both efficacious when used with cotrimoxazole to abrogate the acute disease process in BALB/c mice. Therefore, cefpirome may provide a rational substitute when dealing with ceftazadime resistant strains. In addition, cefpirome may be used for the early management of patients presenting with undiagnosed septicemia due to its broad spectrum of activity.

The BALB/c and C57Bl/6 mouse models of experimental melioidosis have provided researchers with the ability to investigate the pathogenesis of both chronic and acute forms of melioidosis. However, there appear to be differences in the susceptibility of BALB/c mice to infection by *B. pseudomallei* between regions. The resistance of BALB/c mice to *B. pseudomallei* infection has hampered the ability of researchers in North America to utilize this model. Therefore, other mouse models of acute melioidosis may be useful in these areas.

A mouse model of acute experimental melioidosis following i.p. challenge of SWISS outbred mice was also developed recently to investigate the pathophysiological course of *B. pseudomallei* infection (56). The bacterial load of the organs was determined by quantitative bacteriology and by an enzyme-linked immunosorbant assay (ELISA) based on a monoclonal antibody specific to *B. pseudomallei* exopolysaccharide (EPS). The course of *B. pseudomallei* infection in SWISS mice was in accordance with observations in BALB/c mice infected with *B. pseudomallei* via the intravenous route (52),(53),(56). As seen in the studies involving BALB/c and C57Bl/6 mice, *B. pseudomallei* showed a marked organ tropism for the liver and the spleen, with the highest bacterial loads detected in the spleen (56). Electron microscopic examination of the spleen revealed the presence of large numbers of intact bacteria inside of membrane-bound vacuoles of phagocytes which correlates with previous studies demonstrating the ability of *B. pseudomallei* to survive intracellularly (57). Also visible by electron microscopic examination

of the spleen was the presence of a halo surrounding the bacterial cells, which may indicate the expression of capsule *in vivo* (56). This model of acute experimental melioidosis may provide an alternative in cases where susceptible BALB/c mice strains are not available.

Melioidosis may present as an acute or chronic pneumonia, and one of the major routes of infection is through the inhalation of contaminated aerosols. With this in mind, a mouse model of intranasal infection was recently developed using BALB/c and C57Bl/6 mice (58). BALB/c and C57Bl/6 mice were anesthetized with a combination of Hypnorm and midazolam and then inoculated intranasally through one nostril with the appropriate dose of *B. pseudomallei* in 20 μ l of PBS. Liu *et al.*, were able to establish a mucosal infection model of *B. pseudomallei* that was able to spread systemically (58). This model represents a physiologically relevant model of human infection where the route of infection is through inhalation resulting in pulmonary involvement and eventually, septicemia, if the infection is not controlled effectively at mucosal sites. In this study the mouse model of intranasal infection was utilized to investigate factors that contribute to host resistance and to understand the role of mucosal immunity in *B. pseudomallei* infection. The results of this study were similar to the results of the experiments following intravenous inoculation of BALB/c and C57Bl/6 mice in that BALB/c mice demonstrated an increased susceptibility compared to C57Bl/6 mice (58). The authors found that early host resistance correlates with moderate inflammation, while susceptibility correlates with hyperproduction of IFN- γ . In addition C57Bl/6 mice exhibited memory responses with the production of serum IgG and mucosal IgA antibodies to *B. pseudomallei* upon reinfection, indicating the importance of both systemic and mucosal antibodies for protection against disease.

The infection models used for the study of glanders are similar to those for melioidosis. Traditionally hamsters have been utilized for the study of glanders pathogenesis because they are

highly and uniformly susceptible to the disease (40). A Syrian hamster model of intraperitoneal glanders was recently developed in order to further characterize the pathogenesis of glanders and the lesions associated with the infection (59). Syrian hamsters were inoculated i.p. with a lethal dose of *B. mallei* (Budapest strain). Hamsters were sacrificed on days 0-6 and necropsy, histopathology, immunohistochemistry, electron microscopy and bacterial quantitation of the tissues were performed. *B. mallei* was shown to be phagocytosed by peritoneal macrophages and rapidly transported to the mediastinal lymph nodes by the transdiaphragmatic lymphatics. There the bacteria replicated, spilled into the blood through the thoracic duct and spread to the organs. Characteristic glanders lesions were first detected in the spleen, followed by most reticuloendothelial tissues, and eventually in the lung and the brain. This model of infection was found to be suitable for the study of glanders since the pathologic changes observed in the hamster were comparable to that seen in humans. This model was used to demonstrate the role of the *B. mallei* capsule as a major virulence determinant (60). It is also possible to utilize this model for future vaccine and therapeutic trials on glanders.

A mouse model of glanders infection was also sought because hamsters are more susceptible than human beings, and the prevalence of mild subclinical infections in humans is thought to be higher than previously suspected (61). In order to study glanders pathogenesis in an animal model with resistance/susceptibility similar to human beings, Fritz *et al.* chose BALB/c mice and performed a time-course study of histopathologic, ultrastructural, and immunohistologic findings in mice inoculated intraperitoneally with either a lethal or sublethal dose of *B. mallei* (61). BALB/c mice were found to be susceptible to glanders infection and determined to serve as an appropriate model for glanders infection. This model has a number of advantages, including the availability of reagents against cell surface markers, such as cell

phenotype and activation markers, and the availability of genetic variants, such as knockout mice, which could be of significant value in the study of glanders pathogenesis.

An aerosol model of glanders was recently developed based on a similar model used in the study of *Bacillus anthracis* pathogenesis (60). Female BALB/c mice were challenged by aerosol using a whole-body aerosol apparatus. Mice were exposed to a number of inoculum doses of *B. mallei* and monitored for 21 days. The LD₅₀ values were subsequently calculated. This model was also used to study the virulence of a *B. mallei* capsule mutant strain and demonstrate the importance of the *B. mallei* capsule in the pathogenesis of glanders (60). This mouse model of glanders would be particularly useful in the study of glanders pathogenesis and the efficacy of potential therapeutic agents since one of the major routes of infection by *B. mallei* is through inhalation (3).

Non-mammalian infection models such as *Arabidopsis thaliana* and *Caenorhabditis elegans* present an ethical and cost-efficient means of investigating the pathogenesis of bacterial infections. In addition these model systems are genetically tractable which facilitates the study of host-pathogen interactions. With this in mind a number of *Burkholderia* species were tested for the ability to kill the nematode *C. elegans* in order to establish a *C. elegans* infection model (62). The authors found that all strains of *B. pseudomallei* tested were capable of killing the nematodes, likely through an active process involving neuromuscular intoxication. However, *B. thailandensis* was found to be even more efficient at killing *C. elegans* than *B. pseudomallei*, and *B. mallei* was unable to kill the nematode. Therefore, this model was not found to be representative of human infection by these organisms. Another study involving the use of *C. elegans* found that *B. pseudomallei* was lethal to the nematode (63). Similar to the previous study, these authors found that the nemotocidal effects of *B. pseudomallei* on *C. elegans* resulted

from an active process that was likely due to a diffusible toxin. However, these authors found that *B. pseudomallei* and *B. mallei* were lethal to the nematode, while *B. thailandensis* was not. Although the authors attributed the differences in their results to the bacterial strains used and the composition of the growth medium, further studies are required to determine whether *C. elegans* may be an appropriate model host for the study of the pathogenesis of melioidosis and glanders.

We have published a number of manuscripts on the use of animal models to study the pathogenesis of melioidosis and glanders. These are attached as appendices:

Appendix # 14 - Woods, D.E. The Use of Animal Infection Models to Study the Pathogenesis of Melioidosis and Glanders. 2002. Trends Microbiol. 10:483-484.

Appendix # 15 - Lopez, J., Copps, J., Wilhelmsen, C., Moore, R., Kubay, J., St-Jacques, M., Halayko, S., Kranendonk, C., Toback, S., DeShazer, D., Fritz, D.L., Tom, M., and Woods, D.E. 2003. Characterization of Experimental Equine Glanders. Microb. Infect. 5:1125-1131.

IDENTIFICATION AND CHARACTERIZATION OF SURFACE POLYSACCHARIDES AS VIRULENCE DETERMINANTS OF *B. PSEUDOMALLEI* AND *B. MALLEI*

A number of surface polysaccharides have been identified in *B. pseudomallei* and *B. mallei*, and some of these have been implicated as virulence determinants and potential vaccine candidates. One of the surface polysaccharides produced by *B. pseudomallei* is lipopolysaccharide (LPS). The O-antigenic polysaccharide (O-PS) of *B. pseudomallei* LPS is an unbranched heteropolymer with repeating D-glucose and L-talose units with the structure α -D-glucopyranose-(1-3)-6-deoxy- α -L-talopyranose-(1-, in which approximately 33% of the L-talose residues contain 2-O-methyl and 4-O-acetyl substituents, while the other L-talose residues

contain only 2-*O*-acetyl substituents. The O-PS of *B. pseudomallei* was found to be responsible for serum resistance and virulence (31). Serum sensitive mutants of *B. pseudomallei* were identified by transposon mutagenesis and screening for survival in 30% normal human serum. Sequencing of the DNA flanking Tn5-OT182 integrations in three serum-sensitive mutants revealed genes involved in the synthesis of O-PS. The type II O-PS moiety of LPS was found to be absent in the serum-sensitive mutants. A representative serum-sensitive mutant, SRM117, was killed by the alternative pathway of complement and was less virulent in three animal models of melioidosis. SRM117 was approximately 10-fold less virulent than wild type *B. pseudomallei* in the hamster and guinea pig models of melioidosis, and more than 100-fold less virulent in the infant diabetic rat model of melioidosis.

Previous reports have demonstrated that *B. mallei*, *B. pseudomallei*, and *B. thailandensis* are closely related serologically (64). The LPS of *B. mallei* was previously shown to cross-react with polyclonal antibodies raised against *B. pseudomallei* LPS; however, *B. mallei* LPS did not cross-react with a monoclonal antibody (Pp-PS-W) specific for *B. pseudomallei* O-PS indicating that differences existed between *B. mallei* and *B. pseudomallei* O-PS (65). The DNA sequence of the *B. mallei* O-PS gene cluster was found to contain 16 predicted ORFs that were identical to the O-PS genes identified in *B. pseudomallei*, and sequence alignment of the *B. pseudomallei* and *B. mallei* O-PS biosynthetic regions revealed 99% identity at the nucleotide level. The structure of the *B. mallei* O-PS was determined to be 3)- β -D-glucopyranose-(1,3)-6-deoxy- α -L-talopyranose-(1-, in which the talose residue contains 2-*O*-methyl or 2-*O*-acetyl substituents. The *B. mallei* O-PS backbone was found to be similar to that of *B. pseudomallei*, and *B. mallei* O-PS demonstrated *O*-acetyl or *O*-methyl substitutions at the 2' position of the L-talose residue as seen in *B. pseudomallei* O-PS. However, *B. mallei* O-PS was shown to be devoid of an *O*-

acetyl group at the 4' position of the L-talose residue. Recent studies have indicated that the presence of 4-*O*-acetyl groups on the talose residues of *B. pseudomallei* O-PS is due to an *O*-acetylation locus unlinked to the previously described O-PS biosynthetic operon (65). The authors have hypothesized that the unlinked *O*-acetylation locus is either not present or is non-functional in *B. mallei*. The O-PS of *B. mallei* was found to be responsible for serum resistance, similar to the O-PS of *B. pseudomallei* (65). In addition, the insertion sequence element IS407 was identified in the *wbiE* gene of *B. mallei* NCTC 120 and in the *wbiG* gene of *B. mallei* ATCC 15310, two O-PS mutant strains. The presence of IS407 in the O-PS gene clusters of these strains was concluded to be responsible for the loss of expression of O-PS.

Our laboratory recently identified a capsular polysaccharide of *B. pseudomallei* as a major virulence determinant by employing subtractive hybridization between *B. pseudomallei* and a related, non-pathogenic organism *B. thailandensis*. This capsule was found to be necessary for virulence in the Syrian hamster model of acute septicemic melioidosis (14). Insertional inactivation of a glycosyltransferase gene in the capsule locus resulted in a mutant strain that was attenuated for virulence by 10⁵-fold compared to wild type *B. pseudomallei*. The 50% lethal dose (LD₅₀) for wild *B. pseudomallei* is <10 cfu, while the LD₅₀ value for the capsule mutant strain SR1015 was determined to be 3.5 x 10⁵ cfu, similar to that of *B. thailandensis* (6.8 x 10⁵ cfu). Sequencing of the genes involved in the biosynthesis of this polysaccharide revealed open reading frames involved in the synthesis and export of capsular polysaccharides such as the *Escherichia coli* K-10 capsule, the *Haemophilus influenzae* group b capsule, and the capsule produced by *Neisseria meningitidis* serogroup B. The capsule identified by this method was found to be the previously characterized type I *O*-polysaccharide of *B. pseudomallei* with the structure (1,3) linked-2-*O*-acetyl-6-deoxy-β-D-manno-heptopyranose (66). However, due to

genetic homology, the importance of this polysaccharide in virulence, and its high molecular mass, this polysaccharide was concluded to be a capsule (14). This conclusion was further supported by Isshiki *et al.* who separated this polysaccharide from a smooth lipopolysaccharide preparation of *B. pseudomallei* (67). Separation by gel-permeation chromatography and chemical analysis revealed that this polysaccharide is likely a capsule rather than an O-PS component of LPS because it lacks a lipid moiety and was not capable of macrophage activation.

Further studies by our laboratory on the role of this capsule in the pathogenesis of melioidosis have revealed that this capsule is responsible for persistence in the blood through the inhibition of complement factor C3 deposition and phagocytosis (Reckseidler-Zenteno, *et al.*, submitted). Capsule production by *B. pseudomallei* was correlated with increased numbers of organisms in the blood of infected animals, and the addition of purified capsule enhanced the survival of the capsule mutant SR1015 significantly in the Syrian hamster model of acute septicemic melioidosis. The production of this capsule by *B. pseudomallei* was also found to enhance the survival of serum sensitive strains of *B. pseudomallei* by 1000-fold in the presence of normal human serum, likely through the inhibition of complement factor C3b deposition on the bacterial surface, thereby preventing effective opsonization of the bacteria. Western blot and fluorescence microscopy analysis of *B. pseudomallei* incubated in the presence of normal human serum demonstrated that C3b bound the capsule mutant more readily than the wild type. Furthermore, the capsule mutant was phagocytosed more significantly by polymorphonuclear leukocytes compared to wild type *B. pseudomallei*. These results suggest that the production of this capsule contributes to resistance to phagocytosis by inhibiting deposition of C3b on the surface of the bacteria, thereby contributing to persistence of *B. pseudomallei* in the blood of the infected host. This is likely important in the establishment of bacteremia in the host. In addition,

the importance of this capsule in infection was investigated by testing clinical *B. pseudomallei* strains for the production of this capsule. All *B. pseudomallei* strains tested were found to produce capsule, establishing a 100% correlation between capsule production and clinical disease (Reckseidler-Zenteno, *et al.*, submitted).

Other capsular polysaccharides have been identified in *B. pseudomallei*. One of these is a high molecular weight acidic exopolysaccharide with the structure $[\rightarrow 3) - 2 - O - \text{acetyl} - \beta - \text{D} - \text{Galp} - (1 - 4) - \alpha - \text{D} - \text{Galp} - (1 - 3) - \beta - \text{D} - \text{Galp} - (1 - 5) - \beta - \text{D} - \text{KDOp} - (2 \rightarrow)]_n$ which is recognized by patient sera (68). *B. pseudomallei* has been shown to produce two other polysaccharides, one of these was found to be composed of a branched 1,4-linked glucan polymer, and the other was determined to be a triple-branched heptasaccharide repeating unit composed of rhamnose, mannose, galactose, glucose, and glucuronic acid, produced when *B. pseudomallei* was grown in medium containing glycerol (69). The role of these polysaccharides in virulence remains to be elucidated; however, sera from melioidosis patients were strongly reactive to purified exopolysaccharide (70).

A capsular polysaccharide was also identified in *B. mallei* by subtractive hybridization between this organism and *B. thailandensis* (60). The *B. mallei* capsule was shown to be essential for virulence in two animal models. The 50% lethal doses (LD_{50} s) of wild type *B. mallei*, ATCC 23344, and a *B. mallei* capsule mutant, DD3008, in the Syrian hamster model of infection were <10 and $>10^6$ cfu, respectively, representing a $>10^5$ -fold difference in virulence. The capsule mutant was also attenuated for virulence in a mouse model of aerosol infection. The LD_{50} values of ATCC 23344 and DD3008 were 913 and $>10^6$ cfu, respectively, representing a $>10^3$ -fold difference in virulence. Sequencing of the region involved in capsule production revealed a number of genes responsible for the biosynthesis and export of a capsular polysaccharide with strong homology and identical organization to the genes involved in the

production of the *B. pseudomallei* capsule. Like the *B. pseudomallei* gene cluster, the *B. mallei* capsule gene cluster was found to resemble a group 3 capsule gene cluster (60),(14). However, an insertion sequence element, IS407A, was identified downstream of the *B. mallei* capsule cluster that was not found to be associated with the *B. pseudomallei* capsule operon (60). This may suggest that expression of the capsule genes in *B. mallei* are tightly regulated compared to *B. pseudomallei*. It appears that the expression of both capsule and O-PS is regulated in *B. mallei* by the presence of the IS407 element (60),(65). Although the chemical structure of the *B. mallei* capsule has not been deduced, the results presented by DeShazer *et al.* suggest that that it is similar, and possibly identical to the *B. pseudomallei* capsule with the structure α -3)-2-O-acetyl-6-deoxy- β -D-manno-heptopyranose-(1- (60). Although the importance of this capsule in virulence has been established, the role for this capsule in the pathogenesis of glanders has not yet been defined.

We have published a number of manuscripts on the characterization of surface polysaccharides of *B. pseudomallei* and *B. mallei*. These are attached as appendices:

Appendix # 16 - Burtnick, M.N., Brett, P.J., and Woods, D.E. 2002. Molecular and Physical Characterization of *Burkholderia mallei* O-Antigens. J. Bacteriol. 184:849-852.

Appendix # 17 - Brett, P.J., Burtnick, M.N., and Woods, D.E. 2003. The *wbiA* Locus is Required for 2-O-Acetylation of O-Antigens Expressed by *Burkholderia pseudomallei* and *Burkholderia thailandensis*. FEMS Microbiol. Lett. 218:323-328.

Appendix # 18 - Warawa, J., and Woods, D.E. 2002. Melioidosis Vaccines. Expert Rev. Vaccines. 1:477-482.

KEY RESEARCH ACCOMPLISHMENTS

- Developed genetic tools for use in the analysis of the pathogenesis of melioidosis and glanders.
- Developed animal models for the study of melioidosis and glanders.
- Identified and characterized major virulence determinants for *Burkholderia pseudomallei* and *B. mallei*.
- Trained a number of highly qualified personnel.
- Presented our work at international conferences.
- Leveraged the funding from the contract to obtain additional funding to study melioidosis and glanders.

REPORTABLE OUTCOMES

Manuscripts:

1. Woods, D.E., DeShazer, D., Moore, R.A., Brett, P.J., Burtnick, M.N., Reckseidler, S.L., and Senkiw, M.D. 1999. Current Studies on the Pathogenesis of Melioidosis. *Microbes and Infection* 2:157-162, 1999.
2. Deshazer, D., and Woods, D.E. 1999. Pathogenesis of Melioidosis: Use of Tn5-OT182 to Study the Molecular Basis of *Burkholderia pseudomallei* Virulence. *J. Infect. Dis. Antimicrob. Ag.* 16:91-96.
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4. Brett, P.J., and Woods, D.E. 2000. Pathogenesis of and Immunity to Melioidosis. *Acta Trop.* 74:201-210.
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10. Woods, D.E., Jeddelloh, J.A., Fritz, D.F., and DeShazer, D. 2002. *Burkholderia thailandensis* E125 Harbors a Temperate Bacteriophage Specific for *Burkholderia mallei*. *J. Bacteriol.* 184:4003-4017.
11. Woods, D.E. The Use of Animal Infection Models to Study the Pathogenesis of Melioidosis and Glanders. 2002. *Trends Microbiol.* 10:483-484.

12. Warawa, J., and Woods, D.E. 2002. Melioidosis Vaccines. *Expert Rev. Vaccines*. 1:477-482.
13. Brett, P.J., Burtneck, M.N., and Woods, D.E. 2003. The *wbiA* Locus is Required for 2-O-Acetylation of O-Antigens Expressed by *Burkholderia pseudomallei* and *Burkholderia thailandensis*. *FEMS Microbiol. Lett.* 218:323-328.
14. Inglis, T.J.J., Robertson, T., Woods, D.E., Dutton, N., and Chang, B.J. 2003. Flagella-Mediated Adhesion by *Burkholderia pseudomallei* Precedes Invasion of *Acanthamoeba*. *Infect. Immun.* 71:2280-2282.
15. Tribuddharat, C., Moore, R.A., Baker, P., and Woods, D.E. 2003. Characterization of Class A β -Lactamase Mutations of *Burkholderia pseudomallei* That Confer Selective Resistance Against Ceftazidime or Clavulanic Acid Inhibition. *Antimicrob. Ag. Chemother.* 47:2082-2087.
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Abstracts

1. Moore, R.A., Deshazer, D., Reckseidler, S., Weissman, A., and Woods, D.E. Efflux Mediated Aminoglycoside and Macrolide Resistance In *Burkholderia pseudomallei*. Abstr. UA/UC Conference On Infectious Diseases. May, 1998. Banff, Alberta.
2. Reckseidler, S., Moore, RA., Deshazer, D., and Woods, D.E. Identification And Characterization Of Pilin Genes In *Burkholderia pseudomallei*. Abstr. UA/UC Conference On Infectious Diseases. May, 1998. Banff, Alberta.
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7. Reckseidler, S., Moore, R.A., Deshazer, D., And Woods, D.E. Identification and Characterization Of Pilin Genes In *Burkholderia pseudomallei*. International Congress On Melioidosis. November, 1998. Bangkok, Thailand.
8. Burtnick, M.N., and Woods, D.E. Determination Of Genetic Loci Involved In Polymyxin B Resistance In *Burkholderia pseudomallei*. International Congress On Melioidosis. November, 1998. Bangkok, Thailand.
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15. Tribuddharat, C., and Woods, D.E.. Development of a Chronic Infection Model for *Burkholderia mallei*. Canadian Bacterial Diseases Network Annual General Meeting. May, 2000. Canmore, AB.
16. Burtnick, M.N., and Woods, D.E. Implementation of Tn*phoA* and mini-OTn*phoA* Mutagenesis Systems in *Burkholderia pseudomallei* and *Burkholderia thailandensis* Facilitates the Identification of Genes Encoding a Variety of Extracytoplasmic Products. UA/UC Conference On Infectious Diseases. May, 2000. Panorama, B.C.

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21. Reckseidler, S.L., and Woods, D.E. Molecular Characterization of a Group 3 Capsule in *Burkholderia pseudomallei*. Canadian Bacterial Diseases Network Annual General Meeting. May, 2001. Canmore, AB.
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23. Moore, R.A., Tribuddharat, C., and Woods, D.E. Penicillin Resistance in *Burkholderia mallei*. International Congress on Melioidosis. September, 2001. Perth, Australia.
24. Woods, D.E. Pathogenesis and Immunity to *Burkholderia pseudomallei*. International Congress on Melioidosis. September, 2001. Perth, Australia.
25. Woods, D.E. Pathogenesis of Melioidosis. Australian Society for Microbiology. October, 2001. Perth, Australia.
26. Ling, J., Tom, M., Surette, M.G. and Woods, D.E. Environmental Influences on Differential Gene Expression in *Burkholderia pseudomallei*. Interscience Conference on Antimicrobial Agents and Chemotherapy. September, 2002. San Diego, California.
27. Woods, D.E. Glanders and Melioidosis. Interscience Conference on Antimicrobial Agents and Chemotherapy. September, 2002. San Diego, California.

Presentations

1. March, 1998. Washington, D.C. Invited Speaker – Pathogenesis of Melioidosis. USAMIID.
2. April, 1998. Guelph, Ontario. Invited Speaker – Department of Microbiology, University of Guelph.

3. November, 1998. Bangkok, Thailand. International Congress on Melioidosis. Plenary Speaker – Melioidosis Pathogenesis and Vaccine Development.
4. March, 1999. Columbia, Missouri. Invited Speaker – Department of Microbiology and Immunology, University of Missouri.
5. June, 1999. Montreal, Quebec. Canadian Society of Microbiologists. CSM Award Plenary Address – Pathogenesis of Melioidosis.
6. August, 1999. Washington, D.C. Invited Speaker – Pathogenesis of Melioidosis. USAMIIRD.
7. January, 2000. Grahamstown, South Africa – Federation of African Biological Societies - Keynote Speaker – Pathogenesis of and Immunity to Melioidosis.
8. May, 2000. Panorama, British Columbia. UA-UC Conference on Infectious Diseases. Invited Speaker and Conference Organizer.
9. September, 2000. Washington, D.C. Invited Speaker – Genetic Analysis of *Burkholderia spp.* USAMIIRD.
10. November, 2000. Bangkok, Thailand. Invited Speaker-Vaccine Development for Melioidosis. Siriraj Hospital.
11. March, 2001. San Antonio, Texas. Invited Speaker-Department of Microbiology and Immunology. University of Texas Health Sciences Centre at San Antonio.
12. September, 2001. Perth, Australia. Presented Plenary Address at International Congress on Melioidosis.
13. October, 2001. Perth, Australia. Presented Plenary Address at Australian Society for Microbiology Meeting.
14. January, 2002. Toronto, Ontario. CIHR Sponsored Bioterrorism Workshop. Invited Participant.
15. September, 2002. San Diego, California. Invited Speaker, ICAAC. Symposium on Dangerous Bacteria. Glanders and Melioidosis Pathogenesis.
16. December 2002. Ottawa, Ontario. Minister's Advisory Committee on Bioterrorism. Invited presentation on bioterrorism research.
17. December 2002. Los Alamos National Laboratories, Los Alamos, New Mexico. Distinguished Speaker. Pathogenesis of Melioidosis and Glanders.

18. March 2003. Rochester, New York. Invited Speaker-Department of Microbiology and Immunology. University of Rochester.
19. March 2003. Cambridge, England. Invited Speaker-Burkholderia Genomes Workshop. The Wellcome Trust Genome Campus, Hinxton Cambridgeshire.
20. March 2003. Oxford, England. Invited Speaker – Department of Clinical Medicine and Pediatrics. Oxford University.
21. September 2003. Santa Fe, New Mexico. Invited Speaker-Burkholderia Workshop. Department of Homeland Security, USA.
22. September 2004. Singapore. Plenary Speaker – Fourth International Melioidosis Congress.

Database Construction - Genome2Proteome (g2p) Program

The intent of this software is to facilitate the identification of unknown proteins from a given species using the sequenced genome from that species. To do this, we have integrated three pieces of software currently available to molecular biologists, namely: i) an orf (open reading frame) finder, ii) a peptide cutter (e.g. trypsin), and iii) a database search engine to identify the peptide fingerprint best corresponding to a user-inputted “real life” mass spectroscopy fingerprint. Having identified an orf that best fits the user’s data, the user can then use the NCBI BLAST search engine to identify protein homologues if they exist.

This software consists of two phases of analysis. In the first phase, a user-input DNA sequence is prepared/cleaned and 'ripped' into all possible open reading frames (potential proteins) thereby populating a database. Note that this protein database will be an overestimate of the actual translated protein proteome by approximately ten fold, such that no potential protein will go missed, as may be the case in an annotated proteome. Subsequently there is a second analysis phase that accesses this database and performs numerous variations of user-defined analyses on the proteome.

The types of analyses involved in phase two are as follows: each protein in the database undergoes a theoretical digest (breakdown) into specific and predictable fragments (peptides). The user is able to choose the mechanism of this peptide fragmentation (e.g., a trypsin digest). The program then calculates masses of each peptide and populates the database with these values, yielding a peptide fingerprint for each protein entry. Having prepared the database, the user enters “real life” data derived from mass spectroscopy analysis of an unknown protein of interest to the user’s research. The program searches the database with this peptide fingerprint and attempts to match a protein from the database that possesses the same set of values as the query. The results are scored, and it is anticipated that only one strong candidate in the G2P-derived proteome will correspond to the user’s inputted data.

We are currently experiencing an explosion in the number of genomes that have been or are being sequenced, and current annotation efforts are not able to keep up with these genetic data. This program will allow researchers to circumvent the need for an annotated proteome in the

public domain databases and thereby initiate proteomic studies much earlier that would be otherwise possible.

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Canadian Institutes of Health

Canada Research Chair

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Employment or research opportunities applied for and/or received based on experience/training supported by this award

Dr. David DeShazer – USAMIIRD, Ft. Detrick, MD

Dr. Paul Brett – Quorex Pharmaceuticals, San Diego, CA

Dr. Mary Burtnick – Novartis, San Diego, CA

Dr. Chanwit Tribuddharat – Mahidol University, Bangkok, Thailand

Dr. Shauna Reckseidler – Research Associate, Calgary, AB

Dr. Apichai Tuanyok – Research Associate, Calgary, AB

Dr. Richard Moore – Research Associate, Calgary, AB

Dr. Jonathan Warawa – Research Associate, Calgary, AB

Conclusions

The recent advances made by researchers on melioidosis and glanders is indicative of the increasing technology becoming available to the research community. The application of a number of molecular techniques to the study of these organisms combined with increasing advances in immunological and biochemical techniques has provided researchers with the opportunity to reach a clearer understanding of the pathogenesis of these diseases. Sequencing of the genomes of both *B. pseudomallei* and *B. mallei* is currently underway, and the completion of these projects will yield an additional wealth of information regarding virulence determinants, potential vaccine candidates and drug targets.

Although the development of modern techniques has advanced our knowledge of the pathogenesis of infections caused by *B. pseudomallei* and *B. mallei*, there is an increasing need to further understand the diseases melioidosis and glanders in order to develop rapid diagnostics, vaccines and new therapeutics. Both of these diseases are prevalent in a number of regions in the world, and increased travel to these regions may heighten the risk of contracting these diseases. The manifestations of melioidosis and glanders are protean, and the pathogenesis of these infections is very complex. *B. pseudomallei* and *B. mallei* are resistant to a number of antibiotics, and there are no known licensed vaccines available for the prevention of melioidosis and glanders. In addition, both *B. pseudomallei* and *B. mallei* are classified as category B biological agents, which increases the potential for exposure to individuals who would not typically be at risk.

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APPENDICES

Appendix #1 - Reckseidler-Zenteno, S.L., Ling, J., Moore, R.A., and Woods, D.E. 2003.

Recent Research Advances on the Pathogenesis of Melioidosis and Glanders. *Recent Res. Devel. Infect. Immun.* 1:187-202.

Appendix #2 - Brett, P.J., and Woods, D.E. 2000. Pathogenesis of and Immunity to Melioidosis. *Acta Trop.* 74:201-210.

Appendix #3 - Woods, D.E., DeShazer, D., Moore, R.A., Brett, P.J., Burtnick, M.N., Reckseidler, S.L., and Senkiw, M.D. 1999. Current Studies on the Pathogenesis of Melioidosis. *Microbes and Infection* 2:157-162, 1999.

Appendix #4 - Burtnick, M.N., Moore, R.A., Tribuddharat, C., and Woods, D.E. 2000. Antibiotic Resistance Mechanisms of *Burkholderia pseudomallei*. *Res. Adv. Antimicrob. Ag. and Chemother.* 1:1-6.

Appendix #5 - Deshazer, D., and Woods, D.E. 1999. Pathogenesis of Melioidosis: Use of Tn5-OT182 to Study the Molecular Basis of *Burkholderia pseudomallei* Virulence. *J. Infect. Dis. Antimicrob. Ag.* 16:91-96.

Appendix #6 - Deshazer, D., Brett, P.J., Burtnick, M.N., and Woods, D.E. 1999. Molecular Characterization of Genetic Loci Required for Secretion of Exoproteins in *Burkholderia pseudomallei*. *J. Bacteriol.* 181:4661-4664.

Appendix #7 - Bolton, A.J., and Woods, D.E. 2000. A Self-Cloning Minitransposon *phoA* Gene-Fusion System Promotes the Rapid Genetic Analysis of Secreted Proteins in Gram-Negative Bacteria. *Biotechniques.* 29(3):472-474.

Appendix #8 - Reckseidler, S.L., DeShazer, D., Sokol, P.A., and Woods, D.E. 2001. Detection of Bacterial Virulence Genes by Subtractive Hybridization: Identification of the Capsular

Polysaccharide of *Burkholderia pseudomallei* as a Major Virulence Determinant. *Infect. Immun.* 69:34-44.

Appendix #9 - DeShazer, D., Waag, D.M., Fritz, D.L., and Woods, D.E. 2001. Identification of a *Burkholderia mallei* Polysaccharide Gene Cluster by Subtractive Hybridization and Demonstration That the Encoded Capsule is an Essential Virulence Determinant. *Microb. Pathogen.* 30:253-69.

Appendix #10 - Woods, D.E., Jeddeloh, J.A., Fritz, D.F., and DeShazer, D. 2002. *Burkholderia thailandensis* E125 Harbors a Temperate Bacteriophage Specific for *Burkholderia mallei*. *J. Bacteriol.* 184:4003-4017.

Appendix #11 - Tribuddharat, C., Moore, R.A., Baker, P., and Woods, D.E. 2003. Characterization of Class A β -Lactamase Mutations of *Burkholderia pseudomallei* That Confer Selective Resistance Against Ceftazidime or Clavulanic Acid Inhibition. *Antimicrob. Ag. Chemother.* 47:2082-2087.

Appendix # 12 - Burtnick, M.N., Brett, P.J. and Woods, D.E. Identification of Genes Encoding Secreted Proteins Using Mini-*OphoA* Mutagenesis. In: *Methods in Molecular Microbiology*, Vol. 205, *E. coli* Gene Expression Profiles. P.E. Vaillancourt, Ed. pp. 329-338. 2002.

Appendix # 13 - Inglis, T.J.J., Robertson, T., Woods, D.E., Dutton, N., and Chang, B.J. 2003. Flagella-Mediated Adhesion by *Burkholderia pseudomallei* Precedes Invasion of *Acanthamoeba*. *Infect. Immun.* 71:2280-2282.

Appendix # 14 - Woods, D.E. The Use of Animal Infection Models to Study the Pathogenesis of Melioidosis and Glanders. 2002. *Trends Microbiol.* 10:483-484.

Appendix # 15 - Lopez, J., Copps, J., Wilhelmsen, C., Moore, R., Kubay, J., St-Jacques, M., Halayko, S., Kranendonk, C., Toback, S., DeShazer, D., Fritz, D.L., Tom, M., and Woods, D.E. 2003. Characterization of Experimental Equine Glanders. *Microb. Infect.* 5:1125-1131.

Appendix # 16 - Burtnick, M.N., Brett, P.J., and Woods, D.E. 2002. Molecular and Physical Characterization of *Burkholderia mallei* O-Antigens. *J. Bacteriol.* 184:849-852.

Appendix # 17 - Brett, P.J., Burtnick, M.N., and Woods, D.E. 2003. The *wbiA* Locus is Required for 2-O-Acetylation of O-Antigens Expressed by *Burkholderia pseudomallei* and *Burkholderia thailandensis*. *FEMS Microbiol. Lett.* 218:323-328.

Appendix # 18 - Warawa, J., and Woods, D.E. 2002. Melioidosis Vaccines. *Expert Rev. Vaccines.* 1:477-482.

Appendix # 19 - Woods CV.

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Recent research advances on the pathogenesis of melioidosis and glanders

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Abstract

Burkholderia pseudomallei and *Burkholderia mallei* are the etiologic agents for melioidosis and glanders respectively. Melioidosis is recognized as an important health problem in Southeast Asia and Northern Australia, and it poses a concern due to increased travel and military involvement in endemic regions. Isolated cases of melioidosis have been identified in a number of other regions as well. Although glanders has been eradicated from North America and Western Europe, the disease still persists in South America, Eastern Europe, Africa and Asia. In addition, both *B. pseudomallei* and *B. mallei* are classified as category B biological agents, and both have been utilized previously as agents for biological warfare. Due to the importance of these diseases in endemic areas and the fact that the causative agents

are not well characterized on a molecular level, there has been an increased interest in the identification and characterization of virulence determinants in these organisms. This review focuses on the recent advances in the understanding of the pathogenesis of disease caused by B. pseudomallei and B. mallei and describes many of the molecular techniques that have been developed to study these organisms.

Introduction

Burkholderia pseudomallei and *Burkholderia mallei* are gram-negative bacilli that are the causative agents of melioidosis and glanders, respectively. Glanders is primarily a disease of solipeds, but incidental infections may also occur in humans (1),(2). Manifestations of glanders may generally be grouped as acute suppurative infection, acute pulmonary infection, acute septicemic infection and chronic suppurative infection. The incubation period of this disease may range from 1-14 days, depending on the route of infection (3). The prognosis of the localized or chronic forms is favorable; however, the acute septicemic form is usually fatal (3). Melioidosis is described as a glanders-like infection that may affect humans and a variety of animals. Melioidosis may be recognized as inapparent infection, asymptomatic pulmonary infiltration, acute localized suppurative infection, acute pulmonary infection, acute septicemic infection or chronic suppurative infection (3). The incubation period for melioidosis may range from 2 days to 29 years (3),(4). The use of antimicrobials has improved the prognosis for melioidosis; however, even with intensive antibiotic therapy, the mortality rate for the septicemic form is still 40% (5). Infections caused by both *B. pseudomallei* and *B. mallei* are often due to either direct inoculation into wounds and skin abrasions or to inhalation (3),(6),(7),(8). Although these diseases bear a striking resemblance both clinically and pathologically, they are epidemiologically quite different. While *B. pseudomallei* is an environmental saprophyte isolated from soil and water capable of causing disease, *B. mallei* is considered to be an obligate parasite of horses, mules, and donkeys, with no other known natural reservoir (2),(3). The genomes of *B. pseudomallei* and *B. mallei* are highly homologous; however *B. mallei* is considered to be a more refined pathogen due to the smaller size of its chromosome (9).

Melioidosis is recognized as an important health problem in Southeast Asia and Northern Australia, and it poses a concern due to increased travel and military involvement in endemic regions. Isolated cases of melioidosis have been identified in a number of other regions as well, including China, the Indian sub-continent, sub-Saharan Africa, France, Mexico, Brazil, and the Caribbean (7),(10),(11). Although glanders has been eradicated from North America and Western Europe, the disease still persists in South America, Eastern Europe, Africa and Asia (3). In addition, both *B. pseudomallei* and *B. mallei* are classified as category B biological agents, and both have been utilized previously as agents for biological warfare (12). Due to the importance of these diseases and the fact that the causative agents are not well characterized on a molecular level, there has been an increased interest in the identification and characterization of virulence determinants in these organisms in order to develop therapeutic and preventative strategies for the treatment of melioidosis and glanders. This review will focus on the recent advances in understanding the pathogenesis of disease caused by *B. pseudomallei* and *B. mallei*.

Genetic tools for the study of melioidosis and glanders

Much of our increased understanding of melioidosis and glanders has resulted from the rapid increase in studies utilizing molecular biological techniques. As a result of these efforts a variety of molecular tools and methods have been found to be of great use with both *B. pseudomallei* and *mallei*. These tools and techniques are described below.

Signature tagged mutagenesis

Signature tagged mutagenesis (STM) has been used successfully to identify virulence factors in a number of bacterial pathogens. This technique essentially involves infecting an animal with a pool of transposon mutants each containing a unique oligonucleotide tag. Bacteria are then recovered from the animal and the recovered tags compared to the original bank of tags used to infect the animal. Tags that are missing represent mutants which were unable to survive in the host and likely defective in a gene contributing to virulence. In a report by Atkins *et al* (13), signature tagged mutants were subjected to *in vivo* screening in Porton Outbred (PO) mice. A non-capsulated mutant of *B. pseudomallei* was identified which was attenuated in virulence when infection occurred via i.p. infection but was still virulent when challenged via an i.v. route. The acapsular mutant was found to contain an interruption in the mannosyltransferase gene which is required for synthesis of capsular polysaccharide. Interestingly, the gene affected was one of the same capsule biosynthesis genes previously shown as essential for virulence in a hamster model (14). Importantly, this group has demonstrated a means of successfully using STM in *B. pseudomallei*. Although there are no reports yet which describe use of STM in *B. mallei*, it is likely that this procedure could be modified for use in this pathogen as well.

Promoter library and microarray analysis

Expression profiling of pathogenic bacteria allows for the identification of bacterial genes that are differentially regulated in the host. Within this class of genes are those that adapt the organism to host-specific microenvironments or encode virulence determinants. As recovery of organisms from infected host cells are low and contaminated with host genetic material, current attempts to study *B. pseudomallei* virulence gene expression are done *in vitro*. This is based on the assumption that the expression of genes encoding virulence determinants can often be induced *in vitro* by simple modifications of laboratory media (15).

Reporter gene fusions are often used in the discovery and characterization of *cis*-acting promoter regions and *trans*-acting regulatory proteins. In bacterial systems, reporters such as *lacZ* (β -galactosidase), *phoA* (alkaline phosphatase), *cam* (chloramphenicol transacetylase) and *lux* (bacterial luciferase) have been used. Of these systems, the *lux* reporter gene is advantageous as a sensitive, real-time measure of gene expression without disrupting the cell. In addition to identifying and characterizing promoter regions and regulatory proteins, the *lux* reporter system has been used as a marker for monitoring intracellular bacteria within human cells (16) or bacterial virulence gene expression during infection of tissue cell lines or animals (17). The reporter system can also be used in luminescence assays in *in vitro* conditions.

In our laboratory, we are presently attempting to use the *lux* reporter system to identify promoter and regulatory systems that are involved in the expression of virulence

factors. These virulence factors will be investigated further for their pertinence to the pathogenesis of melioidosis caused by *B. pseudomallei*. Briefly, a random promoter library is constructed in *B. pseudomallei* 1026b. The library is then screened for light production when grown under differing growth conditions *in vitro*, that is in conditions that mimic host-specific microenvironments. Our preliminary findings suggest that *B. pseudomallei* is able to respond to extracellular signals such as differing concentrations of iron, magnesium, manganese and glucose, as well as osmotic stress.

In a complimentary approach, we have placed oligonucleotides of a set of known virulence determinants on microarray slides. *B. pseudomallei* 1026b are grown in differing growth condition, such as varying concentrations of iron, glucose and salt, and the RNA isolated to provide quantitative expression data *in vitro*. A similar attempt is underway for microarray expression profiling of known virulence determinants for *B. mallei*.

Subtractive hybridization

Subtractive hybridization has been used successfully to identify an important virulence determinant in *B. pseudomallei* (14). Subtractive hybridization experiments between *B. pseudomallei* and *B. thailandensis* were performed using a PCR-Select Bacterial Genome Subtraction Kit (Clontech) as described by the manufacturer except that hybridization temperature was increased from 63°C to 73°C to account for the high G+C content found in these two species. In these experiments DNA from *B. thailandensis* was used as driver DNA, and DNA from *B. pseudomallei* was used as tester DNA. The PCR products obtained from this procedure were cloned into pZero-2.1 (Invitrogen) and pPCR (18) and enriched for *B. pseudomallei* sequences. The *B. pseudomallei*-specific sequences were then sequenced and the sequences analyzed for homology using the BLASTX program.

Using this approach one clone was identified which had weak homology with WbpX, a glycosyltransferase involved in LPS biosynthesis from *P. aeruginosa*. This gene was insertionally inactivated in wild type *B. pseudomallei* 1026b to generate *B. pseudomallei* SR1015. SR1015 was shown to be capsule deficient and avirulent in a hamster infection model and thereby identified the capsule as an important virulence determinant in *B. pseudomallei*.

Subtractive hybridization has also been used to identify transposable genetic elements in *B. pseudomallei* and in *B. thailandensis* (19). The transposable elements identified in *B. thailandensis* represent the first transposons identified in that species.

Transposon mutagenesis

The use of transposon mutagenesis has proven to an extremely effective means of identifying potential virulence determinants in *B. pseudomallei*. DeShazer *et al.* (20), first described the use of the transposon Tn5-OT182 to isolate motility mutants and to characterize the flagellin structural gene in *B. pseudomallei*. This transposon contains a promoterless β -galactosidase gene which allows for the formation of β -galactosidase transcriptional fusions provided the transposon has inserted downstream of a promoter in the correct orientation. The transposon also contains a *ColE1* origin of replication which allows rapid self-cloning of flanking DNA regions (21) and contains *bla* and *tetAR* genes for selection via ampicillin and/or tetracycline. In *B. pseudomallei* tetracycline at 50

ug/ml is used for selection. *Tn5*-OT182 was successfully used in 6 out of ten *B. pseudomallei* strains tested with up to a tenfold difference in efficiency between strains

Two *Tn5*-based transposons containing truncated *phoA* genes have been used successfully in *B. pseudomallei*, *B. mallei* and *B. thailandensis*. Thus, virulence determinants which are located extracytoplasmically can be identified (22),(23) Use of a *phoA* based transposon required that acid phosphatase negative mutants be constructed (24) using allelic exchange with the gentamycin sensitive strains *B. pseudomallei* DD503 (25) and *B. thailandensis* DW503 (24). *TnPhoA* was delivered to these strains using the vector pRT733; however, the efficiency in *B. mallei* was much less than that found in *B. pseudomallei*. Cloning of flanking DNA was accomplished by digesting transposon containing chromosomal DNA and cloning it into the vector pBR322. Efficiency of this step was low, likely because of the size the transposon (approx. 5kb) and because the cloning vector pBR322 has an effective insert limit of about 7kb. Thus, flanking DNA sizes greater than 2 kb would result in low efficiency at this cloning step.

Construction of another alkaline phosphatase-based transposon, mini-*OphoA* (26) circumvented these problems by incorporating an origin of replication in the transposon thus allowing for self-cloning of flanking DNA regions. As well, transposition of mini-*OphoA* was more efficient than *TnPhoA* in *B. mallei*. Both of these systems have been used successfully in *Burkholderia* spp. for the identification of genes encoding exported proteins.

Atkins *et.al.* (27), utilized a transposon mutagenesis approach to generate a *B. pseudomallei* mutant that was interrupted in the *ilvI* gene which encodes an enzyme essential for branched chain amino acid synthesis. The mutant generated by this approach was significantly attenuated in a mouse model and provided protection against infection by the wild type parent. Unlike the systems described above which rely on conjugation to introduce the transposon into recipient strains, transposon mutants in this study were generated by electroporation of plasmid pUTminiTn5Km2 (28) into *B. pseudomallei* strain 576. Flanking DNA of transposon mutants were sequenced using a PCR protocol (29) which amplified DNA fragments adjacent to the transposon.

Useful vectors for genetic studies of *B. pseudomallei* and *B. mallei*

Allelic exchange allows for the introduction of defined mutations into the chromosome and is a useful method for determining gene function. Allelic exchange involves two successive homologous recombination events between a wild type gene and a cloned gene containing either a deletion or an antibiotic cassette and often employs vectors which contain the conditionally counterselectable marker *rpsL*. This gene confers a streptomycin sensitive phenotype in strains which are streptomycin resistance by virtue of a *rpsL* mutation. Allelic exchange using the *rpsL*-based vector pKAS46 (30) was used to construct *B. pseudomallei* DD503 in which a deletion was introduced into the *amr* locus of *B. pseudomallei* RM101 (25), thus rendering the strain susceptible to aminoglycoside antibiotics and suitable for additional studies involving allelic exchange. Use of pKAS46 with strain DD503 has allowed introduction of defined mutations in the form of deletions and antibiotic resistance cassettes into a variety of genes involving virulence including the flagellin structural gene (*fliC*) (20), serum resistance genes (31) and polymyxin resistance genes (32). *B. pseudomallei* DD503 also allows use of a variety of vectors which employ aminoglycoside resistance as a selective marker which

otherwise could not have been used due to the inherent aminoglycoside resistance found in wild type *B. pseudomallei*.

Vectors known to be useful for complementation of *B. pseudomallei* genes *in trans* include pBBR1Tp (33), pUCP28T, pUCP29T and pUCP31T (34). These vectors allow blue/white selection, contain multiple cloning sites and use trimethoprim or gentamicin resistance as selectable markers. Recently, pBHR1, a mobilizable broad-host-range vector, has been shown to be useful for complementation studies in *B. mallei* (35). Vectors useful for insertional inactivation in *B. pseudomallei* include pSKM11 (36) and pKNOCK-Cm (37) (38). A summary of useful transposons and vectors is shown in Table 1.

Table 1

Transposons	Description	Reference
pOT182	PSUP102(Gm)::Tn5-OT182 Cm ^r Gm ^r Ap ^r Tc ^r	(21)
pUTminiTn5 Km2	Km ^r	(28)
pRT733	oriR6K mobRP4 Tnp ϕ A;Ap ^r Km ^r	(39)
pmini-OphoA	pMB1 oriR, Tn5 tnp, RP4 oriT, ϕ A; Gm ^r	(26)
Vectors		
pKAS46	Mobilizable allelic exchange vector; \square -Dependent R6K replicon; Ap ^r rpsL ⁺ (Sm ^s)	(30)
pKNOCK-Cm	Mobilizable, Broad host range suicide vector Cm ^r	(37)
pSKM11	Mobilizable allelic exchange vector; IncP mob; ColE1 ori; Ap Tc	(36)
p34E-Tp	Cassette cloning vector; Tp ^r	(33)
pBBR1Tp	Mobilizable broad-host-range vector; Tp ^r	(33)
pUCP28T	Broad host range vector: OriT pRO1600 ori; Tp ^r	(34)
pUCP29T	Broad host range vector: OriT pRO1600 ori; Tp ^r	(34)
pUCP31T	Broad host range vector: OriT pRO1600 ori; Gm ^r	(34)
pBHR1	Mobilizable broad host range vector; Km ^r Cm ^r MoBiTec	(35)

Infection models for the study of melioidosis and glanders

A number of animal models have been developed in order to understand the pathogenesis of melioidosis and glanders and to function as systems for assessing the efficacy of antibiotics and vaccine candidates *in vivo*. Also the role of the host in infection can be suggested.

One of the most frequently used animal models for the study of *B. pseudomallei* pathogenesis is the Syrian golden hamster model of acute *B. pseudomallei* infection. It was demonstrated many years prior that a number of animals could be infected with *B. pseudomallei* and the hamster proved to be the most susceptible animal (40). Since

Syrian golden hamsters are exquisitely sensitive to infection by *B. pseudomallei*, a hamster model of acute melioidosis was developed by Brett *et al.* to assess the lethality of a number of *B. pseudomallei* strains (41) (42). Female Syrian golden hamsters, 6-8 weeks of age, were inoculated intraperitoneally (i.p.) with 100 μ l of logarithmic phase cultures adjusted to the appropriate dilution in phosphate-buffered saline. The animals were then monitored for morbidity and mortality, and LD₅₀ values were calculated. When the hamsters were inoculated with those strains predicted to be highly virulent based on secretion profiles, 5/5 of the hamsters in each group succumbed to the infection within 48 hours, while those strains predicted to be less virulent, had no effect on the hamsters. The LD₅₀ values for the highly virulent strains were determined to be <10 cfu, while the LD₅₀ values for the weakly virulent strains were 10⁶ cfu, representing a >10⁵-fold difference in virulence. Bacterial counts from the blood of the animals inoculated with the highly virulent strains displayed a marked bacteremia, while bacteria could not be detected in the blood of the animals inoculated with the lowly virulent strains. The lethal effects produced by the virulent strains were found to be consistent with the clinical manifestations observed in patients with acute septicemic melioidosis (41),(42). This study provided the basis for the classification of non-pathogenic isolates of *B. pseudomallei* as a novel species, *B. thailandensis* (43).

Further studies employing the Syrian hamster model of infection have contributed to the identification of genetic factors responsible for the enhanced virulence of *B. pseudomallei* relative to *B. thailandensis*. A system combining subtractive hybridization between *B. pseudomallei* and *B. thailandensis*, insertional mutagenesis of genes unique to *B. pseudomallei*, and animal virulence studies using the Syrian hamster model was developed by our laboratory in order to identify *B. pseudomallei* virulence determinants (14). As described below, this methodology led to the identification of a capsular polysaccharide as an important virulence determinant in *B. pseudomallei* pathogenesis (14).

The Syrian hamster model of acute melioidosis has been utilized in a number of studies to assess the role of *B. pseudomallei* virulence factors in disease. A number of mutant strains generated by our laboratory have been tested for virulence using this model (14) (20),(25),(31),(42),(44). For example, a number of *B. pseudomallei* Tn5-OT182 mutants susceptible to the bactericidal action of 30% normal human serum and lacking the O-polysaccharide (O-PS) of LPS were found to be less virulent in the Syrian hamster compared to wild type, establishing the importance of O-PS in serum resistance and virulence (31). In contrast, there was no difference in the virulence of a Tn5-OT182 flagellin mutant and type II secretion mutants in this model compared to wild type *B. pseudomallei*, suggesting that these are likely not significant virulence determinants in this animal model (20),(42).

The Syrian hamster model has also been used for investigating the efficacy of antimicrobial agents for the treatment of melioidosis. Subcutaneous injections of sulfadiazine, streptomycin and penicillin were used to treat hamsters infected i.p. with 1-6 x 10³ *B. pseudomallei* (40). Sulfadiazine treatment was effective in preventing death, while streptomycin and penicillin were not. *B. pseudomallei* is relatively susceptible to sulfadiazine but is resistant to streptomycin and penicillin *in vitro* (45) (25). Therefore, there is a correlation between the activity of these antimicrobial agents *in vitro* and *in vivo* using this model (42). There have been no reported studies recently on the activity

of antibiotics using the Syrian hamster model of infection; however, this model would be useful for the evaluation of new antibiotics for the treatment of melioidosis.

Rats have been shown to be relatively resistant to *B. pseudomallei* infection; however, the LD₅₀ of *B. pseudomallei* in infant rats can be lowered significantly by i.p. injection of the diabetogenic compound streptozotocin (STZ) (46),(42). Infant rats weighing 30-40 g were inoculated i.p. with 80 mg of STZ/kg of body weight for 2 consecutive days to induce diabetes. One week after the final injection of STZ, the infant rats were infected with *B. pseudomallei*. The LD₅₀ of *B. pseudomallei* for nondiabetic infant rats was determined to be $>1 \times 10^8$ bacteria, while the LD₅₀ in STZ-induced diabetic infant rats was approximately 1×10^4 bacteria (46). The infection of diabetic infant rats with *B. pseudomallei* resulted in acute septicemic melioidosis and death within 7 days. This animal model was developed because a significant percentage of septicemic melioidosis patients are diabetic, and this model enabled the authors to investigate the effect of insulin on *B. pseudomallei* infection (see 46). This animal model has been successfully utilized in passive immunoprophylaxis studies to demonstrate the protective capacity of antisera raised to purified *B. pseudomallei* O-PS, flagellin protein, and an O-PS-flagellin conjugate, which are potential vaccine candidates for the prevention of melioidosis (47),(48),(49). The infant diabetic rat model has also been useful in assessing the relative virulence of *B. pseudomallei* transposon mutants (20),(31),(44).

As mentioned above, melioidosis may present as an acute pneumonia, an acute septicemia, a chronic infection involving long lasting suppurative abscesses or a subclinical infection that may remain undetected for many years until activated by a traumatic event or a decrease in immunocompetence (50). Recently a number of mouse models have been developed in order to represent the various manifestations of melioidosis and the major routes of infection by *B. pseudomallei*. Previous studies demonstrated that BALB/c mice were highly susceptible to infection with virulent *B. pseudomallei*, while C57Bl/6 mice were found to be relatively resistant (51). Two mouse models of experimental *B. pseudomallei* infection were developed using these inbred strains (52). BALB/c and C57Bl/6 mice were inoculated i.v. in the lateral tail vein with a number of serial dilutions of *B. pseudomallei*, and after 10 days, the LD₅₀ values were calculated. The LD₅₀ values for the BALB/c and C57Bl/6 mice were determined to be 4 cfu and 2.5×10^4 cfu, respectively. The course of infection in BALB/c mice was similar to acute melioidosis, while infection of C57Bl/6 mice was found to mimic chronic melioidosis. Infection of BALB/c mice was rapidly fatal, resulting in bacteremia and death by 96 hours. In contrast, C57Bl/6 mice did not suffer from bacteremia and remained asymptomatic for up to 6 weeks. However, both strains of mice were found to contain high bacterial loads in the liver and spleen. Infection by *B. pseudomallei* in BALB/c and C57Bl/6 mice was further characterized to provide a basis for investigations on the pathogenesis of melioidosis (53). Similar to the previous studies, these authors observed marked organotropism of *B. pseudomallei* for the spleen and liver in both strains of mice, with the highest bacterial load in the spleen. Electron micrographs of the spleen demonstrated intracellular replication of *B. pseudomallei* within membrane-bound phagosomes. In addition, the development of abscesses and granulomatous lesions consistent with those seen in human melioidosis occurred in both the spleen and liver of both mouse strains and was more prevalent in the BALB/c mice

(53). Also BALB/c mice developed a pronounced splenomegaly compared to C57Bl/6 mice, which is observed in acute systemic melioidosis of humans (53). The mechanism for the increased resistance of C57Bl/6 mice to infection of *B. pseudomallei* remains unclear; however, it is thought that the difference may be due to an enhanced innate immune response, possibly effective non-specific bactericidal activity of C57Bl/6 macrophages, a more pronounced Th1-type immune response in the C57Bl/6 mice and an inability of BALB/c mice to contain the infection at sites of inflammation (52),(53),(54).

The BALB/c mouse model of acute disseminated *B. pseudomallei* infection was utilized to study the efficacy of ceftazidime, a fourth generation cephalosporin and potentially effective agent for the treatment of melioidosis (55). The results of this study demonstrated that ceftazidime and ceftazidime are both efficacious when used with cotrimoxazole to abrogate the acute disease process in BALB/c mice. Therefore, ceftazidime may provide a rational substitute when dealing with ceftazidime resistant strains. In addition, ceftazidime may be used for the early management of patients presenting with undiagnosed septicemia due to its broad spectrum of activity.

The BALB/c and C57Bl/6 mouse models of experimental melioidosis have provided researchers with the ability to investigate the pathogenesis of both chronic and acute forms of melioidosis. However, there appear to be differences in the susceptibility of BALB/c mice to infection by *B. pseudomallei* between regions. The resistance of BALB/c mice to *B. pseudomallei* infection has hampered the ability of researchers in North America to utilize this model. Therefore, other mouse models of acute melioidosis may be useful in these areas.

A mouse model of acute experimental melioidosis following i.p. challenge of SWISS outbred mice was also developed recently to investigate the pathophysiological course of *B. pseudomallei* infection (56). The bacterial load of the organs was determined by quantitative bacteriology and by an enzyme-linked immunosorbent assay (ELISA) based on a monoclonal antibody specific to *B. pseudomallei* exopolysaccharide (EPS). The course of *B. pseudomallei* infection in SWISS mice was in accordance with observations in BALB/c mice infected with *B. pseudomallei* via the intravenous route (52),(53),(56). As seen in the studies involving BALB/c and C57Bl/6 mice, *B. pseudomallei* showed a marked organ tropism for the liver and the spleen, with the highest bacterial loads detected in the spleen (56). Electron microscopic examination of the spleen revealed the presence of large numbers of intact bacteria inside of membrane-bound vacuoles of phagocytes which correlates with previous studies demonstrating the ability of *B. pseudomallei* to survive intracellularly (57). Also visible by electron microscopic examination of the spleen was the presence of a halo surrounding the bacterial cells, which may indicate the expression of capsule *in vivo* (56). This model of acute experimental melioidosis may provide an alternative in cases where susceptible BALB/c mice strains are not available.

Melioidosis may present as an acute or chronic pneumonia, and one of the major routes of infection is through the inhalation of contaminated aerosols. With this in mind, a mouse model of intranasal infection was recently developed using BALB/c and C57Bl/6 mice (58). BALB/c and C57Bl/6 mice were anesthetized with a combination of Hypnorm and midazolam and then inoculated intranasally through one nostril with the appropriate dose of *B. pseudomallei* in 20 µl of PBS. Liu *et al.*, were able to establish a

mucosal infection model of *B. pseudomallei* that was able to spread systemically (58). This model represents a physiologically relevant model of human infection where the route of infection is through inhalation resulting in pulmonary involvement and eventually, septicemia, if the infection is not controlled effectively at mucosal sites. In this study the mouse model of intranasal infection was utilized to investigate factors that contribute to host resistance and to understand the role of mucosal immunity in *B. pseudomallei* infection. The results of this study were similar to the results of the experiments following intravenous inoculation of BALB/c and C57Bl/6 mice in that BALB/c mice demonstrated an increased susceptibility compared to C57Bl/6 mice (58). The authors found that early host resistance correlates with moderate inflammation, while susceptibility correlates with hyperproduction of IFN- γ . In addition C57Bl/6 mice exhibited memory responses with the production of serum IgG and mucosal IgA antibodies to *B. pseudomallei* upon reinfection, indicating the importance of both systemic and mucosal antibodies for protection against disease.

The infection models used for the study of glanders are similar to those for melioidosis. Traditionally hamsters have been utilized for the study of glanders pathogenesis because they are highly and uniformly susceptible to the disease (40). A Syrian hamster model of intraperitoneal glanders was recently developed in order to further characterize the pathogenesis of glanders and the lesions associated with the infection (59). Syrian hamsters were inoculated i.p. with a lethal dose of *B. mallei* (Budapest strain). Hamsters were sacrificed on days 0-6 and necropsy, histopathology, immunohistochemistry, electron microscopy and bacterial quantitation of the tissues were performed. *B. mallei* was shown to be phagocytosed by peritoneal macrophages and rapidly transported to the mediastinal lymph nodes by the transdiaphragmatic lymphatics. There the bacteria replicated, spilled into the blood through the thoracic duct and spread to the organs. Characteristic glanders lesions were first detected in the spleen, followed by most reticuloendothelial tissues, and eventually in the lung and the brain. This model of infection was found to be suitable for the study of glanders since the pathologic changes observed in the hamster were comparable to that seen in humans. This model was used to demonstrate the role of the *B. mallei* capsule as a major virulence determinant (60). It is also possible to utilize this model for future vaccine and therapeutic trials on glanders.

A mouse model of glanders infection was also sought because hamsters are more susceptible than human beings, and the prevalence of mild subclinical infections in humans is thought to be higher than previously suspected (61). In order to study glanders pathogenesis in an animal model with resistance/susceptibility similar to human beings, Fritz *et al.* chose BALB/c mice and performed a time-course study of histopathologic, ultrastructural, and immunohistologic findings in mice inoculated intraperitoneally with either a lethal or sublethal dose of *B. mallei* (61). BALB/c mice were found to be susceptible to glanders infection and determined to serve as an appropriate model for glanders infection. This model has a number of advantages, including the availability of reagents against cell surface markers, such as cell phenotype and activation markers, and the availability of genetic variants, such as knockout mice, which could be of significant value in the study of glanders pathogenesis.

An aerosol model of glanders was recently developed based on a similar model used in the study of *Bacillus anthracis* pathogenesis (60). Female BALB/c mice were

challenged by aerosol using a whole-body aerosol apparatus. Mice were exposed to a number of inoculum doses of *B. mallei* and monitored for 21 days. The LD₅₀ values were subsequently calculated. This model was also used to study the virulence of a *B. mallei* capsule mutant strain and demonstrate the importance of the *B. mallei* capsule in the pathogenesis of glanders (60). This mouse model of glanders would be particularly useful in the study of glanders pathogenesis and the efficacy of potential therapeutic agents since one of the major routes of infection by *B. mallei* is through inhalation (3).

Non-mammalian infection models such as *Arabidopsis thaliana* and *Caenorhabditis elegans* present an ethical and cost-efficient means of investigating the pathogenesis of bacterial infections. In addition these model systems are genetically tractable which facilitates the study of host-pathogen interactions. With this in mind a number of *Burkholderia* species were tested for the ability to kill the nematode *C. elegans* in order to establish a *C. elegans* infection model (62). The authors found that all strains of *B. pseudomallei* tested were capable of killing the nematodes, likely through an active process involving neuromuscular intoxication. However, *B. thailandensis* was found to be even more efficient at killing *C. elegans* than *B. pseudomallei*, and *B. mallei* was unable to kill the nematode. Therefore, this model was not found to be representative of human infection by these organisms. Another study involving the use of *C. elegans* found that *B. pseudomallei* was lethal to the nematode (63). Similar to the previous study, these authors found that the nematocidal effects of *B. pseudomallei* on *C. elegans* resulted from an active process that was likely due to a diffusible toxin. However, these authors found that *B. pseudomallei* and *B. mallei* were lethal to the nematode, while *B. thailandensis* was not. Although the authors attributed the differences in their results to the bacterial strains used and the composition of the growth medium, further studies are required to determine whether *C. elegans* may be an appropriate model host for the study of the pathogenesis of melioidosis and glanders.

Identification and characterization of surface polysaccharides as virulence determinants of *B. pseudomallei* and *B. mallei*

A number of surface polysaccharides have been identified in *B. pseudomallei* and *B. mallei*, and some of these have been implicated as virulence determinants and potential vaccine candidates. One of the surface polysaccharides produced by *B. pseudomallei* is lipopolysaccharide (LPS). The O-antigenic polysaccharide (O-PS) of *B. pseudomallei* LPS is an unbranched heteropolymer with repeating D-glucose and L-talose units with the structure -3)-β-D-glucopyranose-(1-3)-6-deoxy-α-L-talopyranose-(1-, in which approximately 33% of the L-talose residues contain 2-O-methyl and 4-O-acetyl substituents, while the other L-talose residues contain only 2-O-acetyl substituents. The O-PS of *B. pseudomallei* was found to be responsible for serum resistance and virulence (31). Serum sensitive mutants of *B. pseudomallei* were identified by transposon mutagenesis and screening for survival in 30% normal human serum. Sequencing of the DNA flanking Tn5-OT182 integrations in three serum-sensitive mutants revealed genes involved in the synthesis of O-PS. The type II O-PS moiety of LPS was found to be absent in the serum-sensitive mutants. A representative serum-sensitive mutant, SRM117, was killed by the alternative pathway of complement and was less virulent in three animal models of melioidosis. SRM117 was approximately 10-fold less virulent

than wild type *B. pseudomallei* in the hamster and guinea pig models of melioidosis, and more than 100-fold less virulent in the infant diabetic rat model of melioidosis.

Previous reports have demonstrated that *B. mallei*, *B. pseudomallei*, and *B. thailandensis* are closely related serologically (64). The LPS of *B. mallei* was previously shown to cross-react with polyclonal antibodies raised against *B. pseudomallei* LPS; however, *B. mallei* LPS did not cross-react with a monoclonal antibody (Pp-PS-W) specific for *B. pseudomallei* O-PS indicating that differences existed between *B. mallei* and *B. pseudomallei* O-PS (65). The DNA sequence of the *B. mallei* O-PS gene cluster was found to contain 16 predicted ORFs that were identical to the O-PS genes identified in *B. pseudomallei*, and sequence alignment of the *B. pseudomallei* and *B. mallei* O-PS biosynthetic regions revealed 99% identity at the nucleotide level. The structure of the *B. mallei* O-PS was determined to be 3)- β -D-glucopyranose-(1,3)-6-deoxy- α -L-talopyranose-(1-, in which the talose residue contains 2-O-methyl or 2-O-acetyl substituents. The *B. mallei* O-PS backbone was found to be similar to that of *B. pseudomallei*, and *B. mallei* O-PS demonstrated O-acetyl or O-methyl substitutions at the 2' position of the L-talose residue as seen in *B. pseudomallei* O-PS. However, *B. mallei* O-PS was shown to be devoid of an O-acetyl group at the 4' position of the L-talose residue. Recent studies have indicated that the presence of 4-O-acetyl groups on the talose residues of *B. pseudomallei* O-PS is due to an O-acetylation locus unlinked to the previously described O-PS biosynthetic operon (65). The authors have hypothesized that the unlinked O-acetylation locus is either not present or is non-functional in *B. mallei*. The O-PS of *B. mallei* was found to be responsible for serum resistance, similar to the O-PS of *B. pseudomallei* (65). In addition, the insertion sequence element IS407 was identified in the *wbiE* gene of *B. mallei* NCTC 120 and in the *wbiG* gene of *B. mallei* ATCC 15310, two O-PS mutant strains. The presence of IS407 in the O-PS gene clusters of these strains was concluded to be responsible for the loss of expression of O-PS.

Our laboratory recently identified a capsular polysaccharide of *B. pseudomallei* as a major virulence determinant by employing subtractive hybridization between *B. pseudomallei* and a related, non-pathogenic organism *B. thailandensis*. This capsule was found to be necessary for virulence in the Syrian hamster model of acute septicemic melioidosis (14). Insertional inactivation of a glycosyltransferase gene in the capsule locus resulted in a mutant strain that was attenuated for virulence by 10^5 -fold compared to wild type *B. pseudomallei*. The 50% lethal dose (LD_{50}) for wild *B. pseudomallei* is <10 cfu, while the LD_{50} value for the capsule mutant strain SR1015 was determined to be 3.5×10^5 cfu, similar to that of *B. thailandensis* (6.8×10^5 cfu). Sequencing of the genes involved in the biosynthesis of this polysaccharide revealed open reading frames involved in the synthesis and export of capsular polysaccharides such as the *Escherichia coli* K-10 capsule, the *Haemophilus influenzae* group b capsule, and the capsule produced by *Neisseria meningitidis* serogroup B. The capsule identified by this method was found to be the previously characterized type I O-polysaccharide of *B. pseudomallei* with the structure (1,3) linked-2-O-acetyl-6-deoxy- β -D-manno-heptopyranose (66). However, due to genetic homology, the importance of this polysaccharide in virulence, and its high molecular mass, this polysaccharide was concluded to be a capsule (14). This conclusion was further supported by Isshiki *et al.* who separated this polysaccharide from a smooth lipopolysaccharide preparation of *B. pseudomallei* (67). Separation by

gel-permeation chromatography and chemical analysis revealed that this polysaccharide is likely a capsule rather than an O-PS component of LPS because it lacks a lipid moiety and was not capable of macrophage activation.

Further studies by our laboratory on the role of this capsule in the pathogenesis of melioidosis have revealed that this capsule is responsible for persistence in the blood through the inhibition of complement factor C3 deposition and phagocytosis (Reckseidler-Zenteno, *et al.*, submitted). Capsule production by *B. pseudomallei* was correlated with increased numbers of organisms in the blood of infected animals, and the addition of purified capsule enhanced the survival of the capsule mutant SR1015 significantly in the Syrian hamster model of acute septicemic melioidosis. The production of this capsule by *B. pseudomallei* was also found to enhance the survival of serum sensitive strains of *B. pseudomallei* by 1000-fold in the presence of normal human serum, likely through the inhibition of complement factor C3b deposition on the bacterial surface, thereby preventing effective opsonization of the bacteria. Western blot and fluorescence microscopy analysis of *B. pseudomallei* incubated in the presence of normal human serum demonstrated that C3b bound the capsule mutant more readily than the wild type. Furthermore, the capsule mutant was phagocytosed more significantly by polymorphonuclear leukocytes compared to wild type *B. pseudomallei*. These results suggest that the production of this capsule contributes to resistance to phagocytosis by inhibiting deposition of C3b on the surface of the bacteria, thereby contributing to persistence of *B. pseudomallei* in the blood of the infected host. This is likely important in the establishment of bacteremia in the host. In addition, the importance of this capsule in infection was investigated by testing clinical *B. pseudomallei* strains for the production of this capsule. All *B. pseudomallei* strains tested were found to produce capsule, establishing a 100% correlation between capsule production and clinical disease (Reckseidler-Zenteno, *et al.*, submitted).

Other capsular polysaccharides have been identified in *B. pseudomallei*. One of these is a high molecular weight acidic exopolysaccharide with the structure $[-\rightarrow 3)-2-O\text{-acetyl-}\beta\text{-D-Galp-(1-4)-}\alpha\text{-D-Galp-(1-3)-}\beta\text{-D-Galp-(1-5)-}\beta\text{-D-KDOP-(2--}\rightarrow)]_n$ which is recognized by patient sera (68). *B. pseudomallei* has been shown to produce two other polysaccharides, one of these was found to be composed of a branched 1,4-linked glucan polymer, and the other was determined to be a triple-branched heptasaccharide repeating unit composed of rhamnose, mannose, galactose, glucose, and glucuronic acid, produced when *B. pseudomallei* was grown in medium containing glycerol (69). The role of these polysaccharides in virulence remains to be elucidated; however, sera from melioidosis patients were strongly reactive to purified exopolysaccharide (70).

A capsular polysaccharide was also identified in *B. mallei* by subtractive hybridization between this organism and *B. thailandensis* (60). The *B. mallei* capsule was shown to be essential for virulence in two animal models. The 50% lethal doses (LD_{50} s) of wild type *B. mallei*, ATCC 23344, and a *B. mallei* capsule mutant, DD3008, in the Syrian hamster model of infection were <10 and $>10^6$ cfu, respectively, representing a $>10^5$ -fold difference in virulence. The capsule mutant was also attenuated for virulence in a mouse model of aerosol infection. The LD_{50} values of ATCC 23344 and DD3008 were 913 and $>10^6$ cfu, respectively, representing a $>10^3$ -fold difference in virulence. Sequencing of the region involved in capsule production revealed a number of genes responsible for the biosynthesis and export of a capsular polysaccharide with

strong homology and identical organization to the genes involved in the production of the *B. pseudomallei* capsule. Like the *B. pseudomallei* gene cluster, the *B. mallei* capsule gene cluster was found to resemble a group 3 capsule gene cluster (60),(14). However, an insertion sequence element, IS407A, was identified downstream of the *B. mallei* capsule cluster that was not found to be associated with the *B. pseudomallei* capsule operon (60). This may suggest that expression of the capsule genes in *B. mallei* are tightly regulated compared to *B. pseudomallei*. It appears that the expression of both capsule and O-PS is regulated in *B. mallei* by the presence of the IS407 element (60),(65). Although the chemical structure of the *B. mallei* capsule has not been deduced, the results presented by DeShazer *et al.* suggest that it is similar, and possibly identical to the *B. pseudomallei* capsule with the structure -3)-2-O-acetyl-6-deoxy- β -D-manno-heptopyranose-(1- (60). Although the importance of this capsule in virulence has been established, the role for this capsule in the pathogenesis of glanders has not yet been defined.

Conclusion

The recent advances made by researchers on melioidosis and glanders is indicative of the increasing technology becoming available to the research community. The application of a number of molecular techniques to the study of these organisms combined with increasing advances in immunological and biochemical techniques has provided researchers with the opportunity to reach a clearer understanding of the pathogenesis of these diseases. Sequencing of the genomes of both *B. pseudomallei* and *B. mallei* is currently underway, and the completion of these projects will yield an additional wealth of information regarding virulence determinants, potential vaccine candidates and drug targets.

Although the development of modern techniques has advanced our knowledge of the pathogenesis of infections caused by *B. pseudomallei* and *B. mallei*, there is an increasing need to further understand the diseases melioidosis and glanders in order to develop rapid diagnostics, vaccines and new therapeutics. Both of these diseases are prevalent in a number of regions in the world, and increased travel to these regions may heighten the risk of contracting these diseases. The manifestations of melioidosis and glanders are protean, and the pathogenesis of these infections is very complex. *B. pseudomallei* and *B. mallei* are resistant to a number of antibiotics, and there are no known licensed vaccines available for the prevention of melioidosis and glanders. In addition, both *B. pseudomallei* and *B. mallei* are classified as category B biological agents, which increases the potential for exposure to individuals who would not typically be at risk.

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Pathogenesis of and immunity to melioidosis

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Abstract

While *Burkholderia pseudomallei*, the causative agent of melioidosis, is becoming increasingly recognized as a significant cause of morbidity and mortality in regions to which it is endemic, no licensed vaccine preparation currently exists for immunization against the disease. Therefore, one of the primary goals of our research has been to identify and characterize antigens expressed by *B. pseudomallei* isolates for the intended purpose of developing a vaccine construct that can be used to actively immunize specific high risk populations against the disease. By utilizing a combination of biochemical, immunological and molecular approaches, our studies now indicate that some of the most promising candidates for this task include flagellin proteins and the endotoxin derived *O*-polysaccharide (PS) antigens expressed by the organism. In this review, we have attempted to summarize the current status of *B. pseudomallei* research while endeavoring to provide a rationale for our approach towards the development of a melioidosis vaccine. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: *Burkholderia pseudomallei*; Melioidosis; Pathogenesis; Virulence factors; Protective antigens; Conjugate vaccine

1. Introduction

Burkholderia pseudomallei, the etiological agent of melioidosis, is a Gram-negative, facultative anaerobic, motile bacillus that is responsible for a broad spectrum of illnesses observed in both humans and animals (Howe et al., 1971; Leelarasamee and Bovornkitti, 1989; Sanford, 1990). While epidemiological surveys have demonstrated that *B. pseudomallei* is endemic to regions which typically border 20° north and south of the equator, the incidence of disease is

particularly high in South-East Asia and northern Australia (Chaowagul et al., 1989; Leelarasamee and Bovornkitti, 1989; Dance, 1991). In north-eastern Thailand alone, an estimated 20% of community acquired septicemia and approximately 40% of deaths due to the complications associated with bacterial sepsis can be attributed to *B. pseudomallei* (Chaowagul et al., 1989). Although the organism is not strictly confined to the equatorial regions, the probability of acquiring melioidosis outside of these geographic domains is exceedingly low (Howe et al., 1971). Since the microbe is nutritionally diverse and is capable of resisting a variety of environmental extremes, it is puzzling as to why a more uniform global distribution of *B. pseudomallei* is not more apparent (Smith et al.,

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1987). The organism can be readily isolated from environmental niches such as rice paddies, still or stagnant waters and moist soils which predominate in the tropics, and it is believed that these habitats are the primary reservoirs from which susceptible hosts acquire infections (Ellison et al., 1969; Leelarasamee and Bovornkitti, 1989).

The manifestations of melioidosis are commonly represented by acute, sub-acute and chronic illnesses, with the clinical indications of some forms of the disease often being mistaken for malaria, plague, pneumonia and miliary tuberculosis (Howe et al., 1971; Smith et al., 1987; Leelarasamee and Bovornkitti, 1989). Resistance to a variety of antimicrobial agents including penicillins, first- and second-generation cephalosporins and many of the aminoglycosides is characteristic of *B. pseudomallei* clinical isolates (Dance et al., 1988; Leelarasamee and Bovornkitti, 1989; Godfrey et al., 1991; Weinberg and Heller, 1997). With this in mind, accurate identification of the organism, evaluation of the severity of the infection and antibiotic susceptibility testing are of paramount importance in devising an effective chemotherapeutic strategy. While the newer therapies that utilize combinations of ceftazidime–cotrimoxazole or amoxicillin–clavulanate for treatment of disease are proving beneficial, the mortality rates associated with the acute septicemic and pulmonary forms of melioidosis are still unacceptably high (Smith et al., 1987; Leelarasamee and Bovornkitti, 1989; Kanai and Kondo, 1994; Weinberg and Heller, 1997; Ho et al., 1997). Typically, prolonged oral therapy is also recommended to assure the full clinical resolution of infections while reducing the potential for recrudescence of disease.

2. Aspects of pathogenesis

Melioidosis is primarily acquired via the inoculation of compromised surface tissues by soils and waters contaminated with *B. pseudomallei*; the highest incidence of disease occurring during the monsoon and rainy seasons (Chaowagul et al., 1989; Leelarasamee and Bovornkitti, 1989). It is believed that during these periods, rising water

tables percolate the organism up through the underlying soils to the surface thus enhancing their potential for exposure to humans and animals. This route of transmission tends to explain the prevalence of disease amongst rice farmers and their families who labor in the rice paddies without the benefit of protective clothing (Chaowagul et al., 1989; Leelarasamee and Bovornkitti, 1989). Another important route of infection appears to be the inhalation and aspiration of contaminated fomites. During the Vietnam war, a disproportionate number of helicopter crewmen succumbed to *B. pseudomallei* infections as compared to other soldiers stationed in the same regions. To explain this phenomenon, it has been proposed that the helicopter rotors acted to disturb infectious dust particles around landing zones and thus facilitated the pulmonary inoculation of the crewmen with *B. pseudomallei* (Howe et al., 1971; Sanfórd, 1990). Alternatively, ingestion of the organism and human to human transmission, although to much lesser extents, have been implicated as routes of inoculation (McCormick et al., 1975). To date, there have been no reports of transmission of disease between animals and humans (Leelarasamee and Bovornkitti, 1989; Dance, 1990).

A number of physiological abnormalities have been correlated with the predisposition of certain populations to *B. pseudomallei* infections. In particular, during a 1 year study of patients admitted to a hospital in north-eastern Thailand for treatment of septicemic melioidosis, 32% demonstrated pre-existing diabetes mellitus (Chaowagul et al., 1989). Similarly, it has been shown in a diabetic infant rat model of infection that such animals are far more sensitive to challenge with *B. pseudomallei* isolates than are the healthy, non-diabetic rats (Woods et al., 1993). The reasons for this increased susceptibility, however, are still being investigated. Other health related factors which appear to increase the probability of acquiring melioidosis include impaired cellular immunity, leukemia/lymphomas, HIV infections, renal disorders, and debilitating afflictions such as alcoholism and parenteral drug abuse (Whitmore and Krishnaswami, 1912; Whitmore, 1913; Leelarasamee and Bovornkitti, 1989; Tanphaichi-

tra, 1989). Although *B. pseudomallei* related illnesses are documented in apparently healthy individuals, the organism is probably still best described as an opportunistic pathogen.

The manifestations of the various forms of melioidosis can be loosely defined as either acute, sub-acute and chronic (Howe et al., 1971). While the incubation periods of *B. pseudomallei* infections are not well defined, a review of the literature does suggest that they can range from as little as a few days to upwards of 26 years (Mays and Ricketts, 1975; Leelarasamee and Bovornkitti, 1989). The acute form of the disease can be subdivided into two groups; the acute pulmonary and the acute septicemic forms. Acute pulmonary symptoms appear rapidly and are characterized by high fever and pulmonary distress. This is followed by the appearance of visceral abscesses and death within a few days if left untreated. The septicemic type illness is also rapidly fatal and displays a high mortality rate when similarly left untreated. Clinical indications of this form include malaise, meningitis, cellulitis, as well as cutaneous and subcutaneous lesions. In many instances the acute manifestations of melioidosis are associated with an appalling mortality rate, even when vigorous chemotherapeutic intervention is implemented (Howe et al., 1971; Leelarasamee and Bovornkitti, 1989; Dance, 1990; Sanford, 1990).

Sub-acute melioidosis is probably best characterized as a prolonged febrile illness. While multiple abscess formation can be observed on the viscera, brain abscesses are seldom found. During the latter stages of the disease, the organism can be readily cultured from blood, pus, urine and other bodily tissues and secretions (Smith et al., 1987; Leelarasamee and Bovornkitti, 1989). In many instances, death occurs within a few weeks to months in the absence of clinical intervention. The sub-clinical or chronic form of the disease is considered to be the most common presentation of melioidosis. It generally remains undiagnosed, however, until activated by a traumatic event or upon post-mortem examination of the tissues (Weinberg and Heller, 1997).

B. pseudomallei causes melioidosis in animals as well as humans. In endemic areas, disease has

been shown to manifest in dogs, cats, rats, rabbits and numerous other species. Animals reported to be immune to this organism are fowl, cattle and water buffalo (Smith et al., 1987). The latter example is rather interesting since these animals are frequently used to pull plows in rice paddies and are, therefore, expected to encounter the microbe on a regular basis. Epizootic outbreaks have been documented in various regions of the world. Notable examples include the death of 24 dolphins in a Hong Kong aquarium in 1975, and an outbreak which appeared in the Caribbean in 1957 resulting in the death of numerous sheep, pigs and goats. Accounts from northern Australia also relate multiple outbreaks of melioidosis in lamb flocks (Sutmoller et al., 1957; Ketterer and Bamford, 1967; Huang, 1976).

Thus, melioidosis should be considered in any febrile patient with a history of residence in a major endemic region. If Gram-negative bipolar staining bacilli are observed in sputum, the organism can be readily cultured and identified (Sanford, 1990). In acute cases, blood and urine cultures are frequently positive, whereas, if chronic or sub-acute forms of the disease are suspected, biopsy may be required. (Sanford, 1990). Serological studies can be helpful for diagnosing active and recrudescing disease, and an immunoglobulin M (IgM) immunofluorescence test is often positive in recent infections (Dance, 1991). Also, indirect hemagglutination and complement fixation and tests are available, but require the testing of paired sera over several weeks to confirm the presence of an active infection (Smith et al., 1987; Chaowagul et al., 1989).

3. Virulence factors and protective antigens

Although *B. pseudomallei* isolates are capable of expressing an impressive array of both secreted and cell-associated antigens, the role(s) of these products in the pathogenesis of disease have to date been relatively ill defined. One of the primary reasons for this has been due to the lack of suitable techniques for genetically manipulating the organism. Due to the recent application of a Tn5 transposon based mutagenesis system for use

in *B. pseudomallei*, we have begun to identify and characterize genetic loci which encode a number of these putative virulence determinants and protective antigens (DeShazer et al., 1997, 1998). Therefore, in the following sections we have attempted to summarize the results of our most current studies, as well as those from other groups, in order to provide an overview of the antigens expressed by this *B. pseudomallei*.

3.1. Secreted antigens

The ability to acquire iron from host sources is a prerequisite for the successful establishment and maintenance of most bacterial infections. Yang et al. have demonstrated that 84/84 *B. pseudomallei* strains examined during their studies tested positive for siderophore production using the chrome azurol S (CAS) assay. A structural and chemical analysis of the siderophore synthesized by *B. pseudomallei* U7 confirmed that the molecule was approximately 1000 Da in size, water soluble with a yellow–green fluorescence and that it belonged to the hydroxamate class (Yang et al., 1991). Furthermore, studies have also demonstrated the siderophore was capable of scavenging iron from both lactoferrin and transferrin in vitro (Yang et al., 1993). The name malleobactin has been proposed for this compound (Yang et al., 1991).

It has been previously shown that *B. pseudomallei* isolates are capable of secreting antigens that demonstrate biological activities consistent with proteases, lecithinases, lipases and hemolysins (Esselman and Liu, 1961; Ashdown and Koehler, 1990; Sexton et al., 1994). However, while these factors have been implicated as important factors in the pathogenesis of the disease, only the protease has been characterized to date. Studies conducted by Sexton et al. have confirmed the presence of a 36 000 kDa antigen with associated proteolytic activities in *B. pseudomallei* culture supernatants. In particular, a protease expressed by *B. pseudomallei* 319a was found to be a metalloenzyme requiring iron for maximal protease activity and demonstrated optimally activity at pH 8.0 and 60°C (Sexton et al., 1994). Furthermore, monoclonal antibodies (Mab) raised against a *Pseudomonas aeruginosa* alkaline protease were

cross-reactive with this antigen (Sexton et al., 1994).

Most recently, via transposon mutagenesis, we have identified an 11.8 kb chromosomal locus in *B. pseudomallei*, that demonstrates a high degree of homology to operons which encode for the products of the main terminal branch of general secretory pathway (GSP) (Pugsley, 1993). Further characterization of the open reading frames in this locus have confirmed that their orientation and physical arrangement are virtually identical to the *pul* gene cluster of *Klebsiella oxytoca* (Pugsley, 1993). Not surprisingly, the phenotypic analysis of the individual transposon mutants has also confirmed their inability to secrete antigens associated with protease, lipase and lecithinase into the extracellular milieu. Interestingly, while we screened more than 30 000 mutants for the loss of one or more of the enzymatic activities, no protease, lipase or lecithinase structural genes were identified. In order to assess the significance of GSP secreted products in the pathogenesis of melioidosis, we compared the virulence of the secretion mutants to the wild type strain in the Syrian hamster model of infection. The results of these studies indicated that while the protease, lipase and lecithinase may play a small role in the pathogenesis of acute melioidosis, mutants deficient in their ability to secrete these particular exoenzymes were not severely attenuated in their ability to cause a fulminating illness (Woods, unpublished data).

In the mid 1950s, several studies demonstrated that filter sterilized *B. pseudomallei* culture supernatants were lethal for mice and hamsters when administered parenterally (Nigg et al., 1955; Heckly and Nigg, 1958; Heckly, 1964). These results were consistent with the fulminating illnesses observed in animals following inoculation with viable bacteria, and suggested that *B. pseudomallei* strains might be capable of secreting a lethal toxin. In studies conducted by Ismail et al. (1987) mouse lethal, thermolabile toxin was reportedly purified to homogeneity and characterized as a 31 000 MW protein. Haase et al. (1997) have also described the presence of cytotoxic activity in culture filtrates. Their results, however, suggest that the antigen is only ~3 kDa in size

and that the cytotoxic activity in this instance is most likely due to the presence of a small peptide. Recently it has been reported that a rhamnolipid purified from *B. pseudomallei* culture supernatants demonstrates a cytotoxic effect against HL60 and HeLa cell lines (Haubler et al., 1998). Since this activity can be neutralized by albumin, however, it is unlikely to be of consequence in the pathogenesis of *B. pseudomallei* infections. Curiously, while it has also been our experience that filter sterilized *B. pseudomallei* culture supernatants are lethal for a variety of tissue culture cell lines, we have been unable to reproduce these effects in animal models, even when using preparations concentrated by lyophilization (Brett et al., 1997, 1998).

3.2. Cell-associated antigens

A number of previous studies have confirmed that *B. pseudomallei* is capable of synthesizing an acid phosphatase. It now appears that the molecule is a glycoprotein with optimal substrate activity around pH 7.2 (Kanai and Kondo, 1991, 1994; Kondo et al., 1996). While it has been reported by Kanai and Kondo (1994) and Kondo et al. (1996) that the acid phosphatase can be readily isolated from both whole cell and supernatant fractions, we have found the enzyme to be predominantly cell-associated (unpublished data). Kanai et al. (1996) have also proposed that the cell-associated form of the enzyme is a high affinity receptor for insulin and that this receptor/ligand interaction may be responsible for modulating the enzymatic profiles of *B. pseudomallei* isolates. Recently, while screening transposon mutants for their inability to hydrolyze the chromogenic substrate 5-bromo-4-chloro-3-indoyl phosphate (X-P), we successfully identified a mutant devoid of acid phosphatase activity (unpublished data). Sequence analysis of the chromosomal DNA flanking the transposon insertion demonstrated the presence of an open reading frame whose translated product was highly homologous to an acid phosphatase expressed by *Francisella tularensis* (var. novicida).

Capsular polysaccharides are a common feature of many bacterial pathogens including

Haemophilus influenzae and *Streptococcus pneumoniae*. These extracellular moieties enable bacteria to evade host defense mechanisms by inhibiting complement activation and phagocytic mediated killing (Joiner, 1988). A review of the literature also indicates that *B. pseudomallei* strains are capable of synthesizing capsular antigens (Smith et al., 1987; Leelarasamee and Bovornkitti, 1989), and that they may play an important role in the pathogenesis of melioidosis. While in vitro studies have determined that encapsulated *B. pseudomallei* strains are as susceptible to phagocytic uptake by polymorphonuclear leukocytes (PMN) as non-encapsulated variants, evidence tends to suggest that the presence of exopolysaccharide confers upon them the ability to resist the bactericidal effects of the phagolysosomal environment (Smith et al., 1987; Pruk-sachartvuthi et al., 1990). This is a feature of *B. pseudomallei* strains that may help to explain why these organisms are capable of remaining latent in a host for upwards of 26 years.

Recently, Steinmetz et al. isolated and purified a high molecular weight capsular antigen (> 150 kDa) from *B. pseudomallei* NCTC 7431 and succeeded in raising a MAb against it (Steinmetz et al., 1995). Via ELISA based techniques, they were able to demonstrate the reactivity of both mucoid and non-mucoid strains with the MAb, thus suggesting that the capsular antigen is constitutively expressed by *B. pseudomallei* strains. Interestingly, temperature appeared to have little effect on the synthesis of the exopolysaccharide since *B. pseudomallei* strains grown at both 15 and 37°C reacted with the MAb. Furthermore, an assay utilizing a variety of *Pseudomonas* and *Burkholderia* spp. as controls was able to confirm the specificity of the MAb for *B. pseudomallei* and *B. mallei* strains only (Steinmetz et al., 1995). More recently, Masoud et al. (1997) have been successful at elucidating the chemical and structural characteristics of a capsular polysaccharide isolated from the virulent clinical isolate *B. pseudomallei* 304b. Their results demonstrated that the exopolysaccharide was a linear unbranched polymer of repeating tetrasaccharide units having the structure (-3)-2-O-Ac- β -D-Galp-(1-4)- α -D-Galp-(1-3)- β -D-Galp-(1-5)- β -D-KDOP-(2-). Similarly,

Nimtz et al. (1997) have demonstrated that a structurally identical capsular antigen is expressed by *B. pseudomallei* NCTC 7431. Studies by both groups have also shown that patient sera reacted strongly with the purified carbohydrate antigens indicating that this carbohydrate polymer is most likely expressed in vivo (Steinmetz et al., 1995; Masoud et al., 1997).

Previous studies have confirmed that the lipopolysaccharide (LPS) antigens expressed by *B. pseudomallei* strains are highly conserved throughout this species (Pitt et al., 1992). In fact, serological evidence suggests that there may be only one serotype of *B. pseudomallei* (Bryan et al., 1994). In order to investigate this phenomenon, Perry et al. has characterized the LPS antigens isolated from a number of *B. pseudomallei* strains (Perry et al., 1995). Their results demonstrated that *B. pseudomallei* strains coordinately express two distinct somatic O-antigens (PS) on their cell surface. The Type I antigen consists of a high-molecular weight unbranched 1,3-linked homopolymer of 2-O-acetylated 6-deoxy- β -D-manno-heptopyranosyl residues, while the Type II antigen is an unbranched heteropolymer consisting of (-3)- β -D-glucopyranose-(1-3)-6-deoxy- α -L-talopyranosyl-(1-disaccharide repeats (L-6dTalp: ~33% O-4 acetylated and O-2 methylated; ~66% O-2 acetylated) (Knirel et al., 1992; Perry et al., 1995). While the simultaneous expression of two or more LPS moieties is not an uncommon feature associated with Gram-negative bacteria, the degree to which the two PS antigens are conserved amongst *B. pseudomallei* strains is quite remarkable (Perry et al., 1995).

It has been previously reported that *B. pseudomallei* strains are resistant to the bactericidal effects of normal human serum (Ismail et al., 1988). Recently, we have established that the presence of Type II PS is essential for conferring this resistance phenotype. Via the application of the transposon mutagenesis system, we have identified a number of mutants that demonstrate a marked sensitivity, in comparison to the parental strain, to the bactericidal effects of normal serum. Sequence analysis of the chromosomal DNA flanking these transposon insertions has enabled us to identify a 17.5 kb region of the chromosome that

is required for the synthesis of the Type II antigen and conferring the serum resistance phenotype (DeShazer et al., 1998). Using the infant diabetic rat model, we have also confirmed that Type II PS is probably a significant determinant in the pathogenesis of melioidosis since the LD50 value associated with a Type II PS mutant is approximately 140 fold higher than that of the wild type strain (Woods et al., 1993; DeShazer et al., 1998).

Flagella are commonly recognized as important virulence determinants expressed by bacterial pathogens since the motility phenotype imparted by these organelles often correlates with the ability of an organism to cause disease (Penn and Luke, 1992; Moens and Vanderleyden, 1996). Therefore, we have focused a great deal of attention towards determining the relative importance of motility in the pathogenesis of melioidosis. In previous studies we have demonstrated that a significant degree of size and antigenic homogeneity exists amongst flagellins expressed by *B. pseudomallei* isolates. Furthermore, we have also shown that flagellin specific antiserum is capable of passively protecting diabetic infant rats against a *B. pseudomallei* challenge (Brett et al., 1994). Curiously, however, in recent studies we have found that there was no significant difference between the virulence capacities associated with a wild-type strain of *B. pseudomallei* and non-motile mutants in either the diabetic infant rat or Syrian hamster models of infection (DeShazer et al., 1997). Thus, taken together, these results indicate that while flagella and/or motility may not be major virulence determinants in the pathogenesis of melioidosis, purified flagellin may still serve as a protective immunogen against *B. pseudomallei* infections.

4. Conjugate vaccines

Previous studies have demonstrated that both polyclonal antiserum and MAb's raised against *B. pseudomallei* flagellin proteins, LPS and a tetanus toxin-PS glycoconjugate are capable of passively immunizing diabetic infant rats against challenge with *B. pseudomallei* (Brett et al., 1994; Bryan et al., 1994). While these initial results were encour-

aging, it had become evident to us, however, that an active vaccine preparation would be the most practical for immunizing high risk populations against melioidosis. Therefore, with this in mind, we synthesized a glycoconjugate molecule that incorporated both flagellin protein and PS antigens. Based upon the preliminary success of these studies, we believe that this preparation is a reasonable vaccine candidate for actively immunizing against the disease (Brett and Woods, 1996).

It has been well documented that T cell-independent type 2 (TI-2) antigens such as bacterial polysaccharides are capable of eliciting protective antibody responses in adults (Dintzis, 1992; Jennings, 1992), but act poorly as immunogens in the elderly, in immunocompromised populations and in children less than 18 months of age (Gold et al., 1977; Robbins, 1978; Jennings, 1983). In adult populations, TI-2 antigens typically stimulate the synthesis of antigen specific immunoglobulin M (IgM), but often fail to evoke augmented immune responses and isotype conversions following boosting with carbohydrate preparations. This phenomenon is primarily due to the inability of activated B lymphocytes to recruit CD4⁺ T cell (Th) involvement via major histocompatibility complex type II (MHC II) restricted events (Hodgkin and Basten, 1995; Mond et al., 1995). In order to remedy this situation, a number of bacterial carbohydrates (such as capsular polysaccharides or somatic O-antigens) have been covalently coupled to protein or peptide carrier molecules in order to facilitate T cell-dependent (TD) immune responses against the particular carbohydrate moieties (Cryz et al., 1986; Insel and Anderson, 1986; Winter et al., 1988; Watson et al., 1992; Fattom et al., 1993; Konadu et al., 1994; Lett et al., 1994). The presence of T cell epitopes inherent to peptide or proteinaceous carriers is believed to facilitate these events (Braley-Mullen, 1980).

Therefore, by conjugating the PS moieties to the flagellin carrier, we have been able to elicit augmented immune responses against the PS components of the vaccine while concomitantly evoking desirable immunoglobulin (Ig) class switching events (Brett and Woods, 1996). More importantly, the use of an active vaccine containing

only the PS portion of LPS and not the toxic component of LPS, namely lipid A, conjugated to flagellin protein obviates the toxic side effects of LPS but takes advantage of the protective response to the PS moiety. Furthermore, the coordinate presentation of multiple protective antigens has served to enhance the immunological repertoire of the vaccine recipient while providing immunity against a number of *B. pseudomallei* strains.

Since the size of the saccharide components displayed by a conjugate molecule appears to influence the immunogenicity of the preparation, a heterogeneous combination of PS antigens were covalently linked to the flagellin protein carrier (Dintzis et al., 1983, 1985; Jennings, 1992). The incorporation of larger PS fragments act to increase the size of the conjugate molecules such that the cross-linking of B cell mIg's is maximized. At the same time, the larger PS molecules also tend to retain conformationally stabilized epitopes which appear to be critical in the design of a successful vaccine (Jennings, 1992). The smaller oligosaccharide fragments appear to be critical for stimulating immunoglobulin responses against terminal epitopes of the carbohydrate molecules.

We have demonstrated that immune serum raised against the glycoconjugate conjugate reacts with both purified flagellin protein and both Type I and II PS; a situation that is obviously critical if the immune serum is to be effective against a *B. pseudomallei* challenge. This confirmed that native epitopes displayed by the precursor molecules were maintained during the synthesis of the conjugate molecule. Furthermore, passive immunization studies have demonstrated that the IgG fraction purified from the immune serum was capable of protecting diabetic infant rats from a challenge with *B. pseudomallei* (Brett and Woods, 1996). Based upon the success of these preliminary studies, we are currently characterizing the efficacy of this vaccine preparation in active immunization studies.

Since *B. pseudomallei* isolates have been shown to express both the Type I and II PS antigens (an exception being 824a which displays only the Type I antigen) and the flagellin proteins appear

to be antigenically conserved, we expect the conjugate molecule to afford protection against the majority of *B. pseudomallei* strains that we will encounter in our future studies.

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Review

Current studies on the pathogenesis of melioidosis

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ABSTRACT – *Burkholderia pseudomallei* is a major cause of bacterial septicemias in many parts of the world, particularly Thailand; the known geographic range of the organism appears to be enlarging as awareness of the organism and the disease it causes – melioidosis – increases. *B. pseudomallei* is intrinsically resistant to most antibiotics, and our knowledge of *B. pseudomallei* pathogenesis is lacking. Thus, the long-term objective of our research is to define at a molecular level the pathogenesis by combining genetic, immunologic, and biochemical approaches with animal model studies. Basic studies on *B. pseudomallei* pathogenesis are acutely needed to provide a knowledge base to rationally design new modes of therapy directed against this organism. © Elsevier, Paris

melioidosis / *Burkholderia pseudomallei* / pathogenesis / molecular biology

1. Introduction

Burkholderia pseudomallei is a common cause of human pneumonia and fatal bacteremias in endemic areas [1]. The organism is an opportunistic pathogen, and those individuals particularly susceptible to *B. pseudomallei* infection have underlying conditions such as diabetes or renal disease [1, 2]. Clinical manifestations of *B. pseudomallei* infection, a disease known as melioidosis, vary greatly from an asymptomatic state, to benign pneumonitis, to acute or chronic pneumonia, or to overwhelming septicemia [3]. Treatment of melioidosis can involve up to nine months of antibiotic therapy [4], and relapse of disease is common. Additionally, the latency period of the organism may vary from two days to 26 years [3, 5].

B. pseudomallei occurs as a soil organism in Southeast Asia and northern Australia, and incidents of melioidosis are generally confined to these endemic areas [1]. Recent surveys, however, show that the organism is much more prevalent worldwide than previously believed, and isolation of *B. pseudomallei* from the environment and in clinical situations in parts of Africa, the Middle East, Europe, and in Central and South America has been documented [6]. Humans as well as wild and domestic animals residing in the areas subtended by latitude 20° north to 20° south have evidence of subclinical and clinical disease [6], and as many as 10 to 30% of Southeast Asians have serum antibodies to the organism. Likewise, individuals who travel to or through, or who reside in, endemic areas are susceptible to infection. One to two percent of healthy soldiers and up to 9% of wounded

individuals who served in Vietnam are serologically positive. Thus, the approximately three million American soldiers who traveled and lived in that endemic region include a significant number who have latent infection, often recrudescing after a latent period of months to years [7]. More recently, a report from India indicates that *B. pseudomallei* is present in that country and that it is responsible for a significant level of disease [8].

Prior to the use of antimicrobials, the case fatality rate from apparent *B. pseudomallei* infections was 95%. French experience in Indochina indicated that with chloramphenicol therapy the mortality was 20%. With better diagnosis and more prolonged appropriate therapy, mortality in forms other than septicemic should be low [1, 9]. Even with vigorous appropriate antibiotics and supportive therapy, the mortality in patients with melioidosis septicemia is 40% or greater.

In July 1990, our laboratory initiated a collaborative effort with scientists in Thailand to define the pathogenesis due to *B. pseudomallei*. Melioidosis has been recognized as the major cause of bacterial septicemia in Thailand. Our principal collaborators in Thailand include Dr. Nicholas J. White (Director of the Wellcome-Mahidol-Oxford Tropical Medicine Research Programme, Hospital for Tropical Diseases, Bangkok, Thailand), and Dr. Wipada Choawugul (Chairman of the Department of Medicine, Sappasitprasong Hospital, Ubon Ratchatani, Thailand). They have been instrumental in supplying us with clinical isolates of *B. pseudomallei*, patient sera, and clinical information on melioidosis patients. This collaboration has been a tremendously fruitful one resulting in the acquisition of important new information regarding the pathogenesis of *B. pseudomallei* disease.

* Correspondence and reprints

2. Animal models of melioidosis

A significant advance in our understanding of *B. pseudomallei* pathogenesis has been provided by the development in our laboratory of an animal model of infection due to this organism. An intriguing observation regarding melioidosis is that a significant percentage of patients who develop the disease have preexisting diabetes mellitus. In this regard, we tested the hypothesis that insulin may modulate the growth of *B. pseudomallei*, and we demonstrated that insulin markedly inhibits the growth of *B. pseudomallei* in vitro and in vivo. The growth rate of *B. pseudomallei* in minimal medium containing human recombinant insulin was significantly lower than that of control cultures containing no insulin. *B. pseudomallei* grew at an increased rate in sera obtained from diabetic rats compared with sera obtained from control animals. When the insulin level was restored by the addition of human recombinant insulin, the growth rate was reduced to a level similar to that seen in control sera. *B. pseudomallei* also grew significantly better in insulin-depleted human serum than in control human serum. ¹²⁵I-insulin binding studies demonstrated that *B. pseudomallei* possesses a specific, high-affinity binding site for human insulin. In in vivo studies, rats made diabetic by streptozotocin injection (80 mg/kg of body weight, intraperitoneally) were significantly more susceptible to *B. pseudomallei* septicemia than control rats [2]. More recently, we used a model of *B. pseudomallei* infection in Syrian golden hamsters which has proven to be tremendously useful in our studies on the pathogenesis of infections due to *B. pseudomallei* [10]. The development of these animal models has allowed us to proceed with a number of immunoprotection studies. Additionally, the animal models have allowed us to assess the role of a number of putative virulence determinants [11].

3. Genetic tools

One of the most exciting developments to arise involving the study of *B. pseudomallei* pathogenesis has been the ability to employ Tn5-OT182 mutagenesis in this species [12, 13]. This has greatly enhanced our understanding of the roles of specific virulence determinants when utilized in conjunction with animal models of infection. Tn5-OT182 is well characterized genetically, and its entire nucleotide sequence has been determined [12]. Tn5-OT182 contains *bla* and *tetAR*, the genes encoding ampicillin resistance (Ap^r) and tetracycline resistance (Tc^r), respectively. Most *B. pseudomallei* isolates are inhibited by tetracycline at a concentration of 12.5 µg/mL; thus, the use of tetracycline at a concentration of 50 µg/mL efficiently selects for those cells that have obtained a copy of Tn5-OT182 in the chromosome. Tn5-OT182 is a self-cloning, promoter probe transposon that integrates randomly in the *B. pseudomallei* chromosome [12, 13]. This transposon contains a *ColE1* origin of replication, allowing for the rapid cloning of flanking DNA from the desired mutant by digesting chromosomal DNA with the appropriate restriction endonuclease, ligating and transforming *Escherichia coli* with selection for ampicillin and/or tetra-

cycline. In addition, Tn5-OT182 contains a promoterless *lacZ* at one end, allowing for the formation of transcriptional fusions when the transposon integrates downstream of a promoter [12, 13]. This feature is useful for determining the orientation of a mutated gene as well as identifying physical and/or chemical stimuli that may affect expression of the mutated gene.

The ability to introduce defined mutations into the *B. pseudomallei* chromosome by allelic exchange would be a powerful method for studying genetic determinants of this organism, especially those encoding virulence factors. No methods or strains currently exist for allelic exchange in this organism. Allelic exchange involves two successive homologous recombination events between a wild-type chromosomal gene and a cloned mutant allele [14]. Several allelic exchange vectors contain the conditionally counterselectable marker *rpsL*, which confers a streptomycin-sensitive (Sm^s) phenotype and serves as a positive selection for mutants that have undergone a double crossover and have lost vector sequences [14-16]. Allelic exchange vectors based on *rpsL* can only be used in strains that are resistant to streptomycin via a point mutation in the chromosomal *rpsL* gene, which encodes the ribosomal protein S12 [14]. It is not possible to select for a Sm^r *rpsL* mutant of *B. pseudomallei*, as it displays a high level of resistance to streptomycin by the multidrug-resistance pump encoded by the *amrRABopA* genes (see below). We have utilized the *B. pseudomallei* strain RM102, which is Sm^s due to the *amrA*::Tn5-OT182 mutation, to select for a strain that is Sm^r due to an *rpsL* mutation. The *amrRABopA* locus was subsequently deleted from this strain using the allelic exchange vector pKAS46 [16]. The resulting strain, DD503, has been used successfully in allelic exchange experiments with pKAS46.

We have also constructed a trimethoprim-resistance (Tp^r) cassette vector termed p34E-Tp [17]. The Tp^r cassette is small (approximately 600 bp) and is a useful selective marker for cassette mutagenesis of cloned genes and allelic exchange experiments in *B. pseudomallei*. This Tp^r cassette was used to construct the broad-host-range plasmids pBBR1Tp [17] and pUCP28T/pUCP29T [19]. These plasmids replicate in *B. pseudomallei* and have been used as cloning vectors in this organism for the complementation of mutated genes in trans [12, 17-19].

4. Serum resistance

The alternative and classical complement pathways play an integral role in the elimination of invasive microbial pathogens by the generation of opsonic, chemotactic, and lytic functions [20, 21]. Many Gram-negative bacteria are susceptible to the serum bactericidal system, but some pathogens possess mechanisms to evade killing by this system. The ability to evade complement-mediated killing is considered to be an important virulence determinant of invasive Gram-negative bacteria [20, 21].

Resistance to complement-mediated bacteriolysis is probably a key virulence determinant of *B. pseudomallei*, since 50-70% of melioidosis patients are bacteremic on admission to the hospital [22]. Ismail et al. demonstrated that clinical and environmental isolates of *B. pseudomal-*

B. pseudomallei are highly resistant to killing by 10–30% normal human serum (NHS) [23]. Recently, Egan and Gordon have found that *B. pseudomallei* is resistant to killing by 50% NHS [24]. We have recently identified a molecular determinant required for the serum resistance phenotype of *B. pseudomallei* (DeShazer, unpublished). We found that *B. pseudomallei* multiplies in 10–30% NHS, and a screen was developed for the identification of mutants that did not multiply in 30% NHS. Approximately 1 200 Tn5-OT182 mutants were screened, and three serum-sensitive mutants were identified. The type II O-antigenic polysaccharide (O-PS) moiety of lipopolysaccharide (LPS) [25] was not present in the serum-sensitive mutants. A representative serum-sensitive mutant, SRM117, was killed by the alternative pathway of complement and was less virulent than the parental strain in hamsters, infant diabetic rats, and guinea pigs. The Tn5-OT182 integrations in the serum-sensitive mutants were physically linked on the *B. pseudomallei* chromosome, and further genetic analysis of this locus revealed a cluster of 15 genes required for the biosynthesis of the type II O-PS moiety of LPS. The proteins encoded by these genes were similar to proteins involved in bacterial polysaccharide biosynthesis. We conclude from these results that the type II O-PS moiety of LPS is required for serum resistance and virulence.

5. Secretion of exoproducts

Clinical isolates of *B. pseudomallei* produce several hydrolytic enzymes that are secreted into the extracellular milieu, including lecithinase, lipase, and protease [26]. The role of these secreted products in pathogenesis is currently unknown, but their mechanism of secretion has recently been elucidated (DeShazer, unpublished). Twenty-nine unique Tn5-OT182 mutants unable to secrete lecithinase, lipase, or protease were identified on 3% skim milk plates and egg yolk agar plates. The Tn5-OT182 integrations in these mutants were mapped to two distinct loci. Twenty-six of the mutations were clustered at a genetic locus spanning approximately 12 kb. The DNA sequence of this region consists of 11 genes, *gspDEF-GHIJKLMN*, which encode proteins with a high degree of homology to general secretory or type II secretion pathway proteins from a variety of Gram-negative bacteria [27]. In addition, three mutations were identified within an unlinked gene (*gspO*) which encoded a protein with homology to type IV prepilin peptidases. The majority of the lecithinase, lipase, and protease produced by the secretion mutants was found in the cell-associated fractions rather than in the supernatants. Taken together, the results of these studies suggest that protease, lipase, and lecithinase are secreted by a type II secretion pathway in *B. pseudomallei*.

6. Adherence

Adhesion is a fundamental requirement for many bacteria in order to colonize the host and cause disease. The role of adhesion in *B. pseudomallei* infection is still undefined; however, recently our laboratory has initiated studies to investigate the involvement of pili in adherence and infection.

One of the more common manifestations of melioidosis is a pulmonary infection that may be acute or chronic. Previous case report studies have shown that it is possible to contract *B. pseudomallei* through inhalation of dust contaminated with the organism [28]. Experiments have shown that animals can be infected with *B. pseudomallei* via the oral or nasal mucosa or by ingestion [29]. In addition, *B. pseudomallei* has been shown to adhere to buccal epithelial cells by our lab and others [3]. This evidence suggests that *B. pseudomallei* can colonize mucosal tissue and cause infection.

Many pathogenic bacteria mediate adhesion through fimbriae or pili [30]. Our laboratory has evidence to suggest that *B. pseudomallei* possesses at least two types of pili. Electron microscopy studies by this laboratory have demonstrated the presence of different types of pili, which do not appear to be coexpressed (Reckseidler et al., unpublished). We have cloned and sequenced a gene (*fimA*) encoding a 15-kDa pilin structural protein that exhibits homology to type I pili [30], and we have also cloned accessory genes involved in the assembly of type I pili: FimC, a chaperone protein, and FimD, an outer membrane usher protein (Reckseidler et al., unpublished). This organism also contains the genes involved in the production of type IV pili [30]. We have cloned genes with strong homology to *pilB*, *pilC*, and *pilD* of *Pseudomonas aeruginosa* (Reckseidler et al., unpublished). Gene replacement techniques are currently under way to define the role of these genes in adhesion. Our future aims are to define the role of pili in adhesion and to correlate adherence with pathogenicity.

7. Intracellular survival

One interesting aspect of the clinical progression of melioidosis is the recrudescence of the disease. There have been reported cases of reactivation of latent *B. pseudomallei* infections into acute, fulminating, and sometimes fatal infections up to 26 years after the initial infection [5]. Ribotype analysis has demonstrated that in many cases relapse results from reactivation of a persistent endogenous source of infection [31, 32]. We have postulated that *B. pseudomallei* can persist in the intracellular environment of eukaryotic cells in a dormant stage until reactivated. Relapse or recurrence of infection when the course of treatment is not long enough and difficulties in antimicrobial therapy despite in vitro susceptibility suggest that intracellular survival is important in the pathogenesis caused by this organism. We have initiated studies to define the roles of invasion and intracellular survival during the course of *B. pseudomallei* infection.

Our studies in collaboration with Dr. Terry Beveridge of the University of Guelph (Ontario, Canada) have demonstrated that *B. pseudomallei* can invade and replicate intracellularly in a number of cell types. *B. pseudomallei* can invade both cultured epithelial cell lines, such as HeLa, CHO, A549, and Vero, and professional phagocytic cells, such as rat macrophages and human polymorphonuclear neutrophils. Electron microscopic visualization of infected HeLa, polymorphonuclear neutrophils, and U937 cells confirmed the presence of intracellular bacte-

ria localized in membrane-bound vacuoles. *B. pseudomallei* has been shown to replicate intracellularly in polymorphonuclear neutrophils at 16 h and in epithelial A549 cells up to 48 h. This pathogen is resistant to the cationic peptide protamine sulfate and purified human defensin HNP-1 (found in lysosomes). Resistance to antimicrobial activity may facilitate intracellular survival [33].

We have identified a mutant, AJ1D8, derived from *B. pseudomallei*, that invades A549 cells at only 11% of the level of the parent strain and we have begun characterization. The DNA flanking the Tn5-OT182 insertion was found to share considerable homology with *Pseudomonas syringae* CopR, *E. coli* PcoR, *Streptomyces coelicolor* Afq1 and *Enterococcus faecium* VanR. These proteins are transcriptional activators of two-component signaling systems whereby signal transduction is accomplished by a phosphotransferase system. These results suggest that AJ1D8 harbors a mutation in a transcriptional activator protein of a two-component regulatory system that may be involved in regulating invasion of *B. pseudomallei* into epithelial cells [20].

Studies are currently under way to determine possible mechanisms of intracellular survival. These studies involve two main areas, the occurrence of phagosome-lysosome fusion and physiological response of *B. pseudomallei* to low pH environments. Current data indicate that this pathogen produces an extracellular polysaccharide in response to low pH challenge (Senkiw and Woods, unpublished).

8. Antibiotic resistance

The successful treatment of melioidosis patients is difficult because *B. pseudomallei* is inherently resistant to a variety of antibiotics including beta-lactams, aminoglycosides, macrolides, and polymyxins [28, 34]. We have utilized Tn5-OT182 mutagenesis to identify genes involved in aminoglycoside and polymyxin resistance. Initial experiments designed to identify genes involved in aminoglycoside resistance resulted in the isolation of two transposon mutants, *B. pseudomallei* RM101 and RM102, which demonstrated an eight- to 128-fold increase in susceptibility to the aminoglycosides streptomycin, gentamicin, neomycin, tobramycin, kanamycin, and spectinomycin. Further examination revealed that the mutants, in contrast to the parent strain, were also susceptible to the macrolides erythromycin and clarithromycin but not to the lincosamide clindamycin. Sequencing of DNA flanking the transposon insertions revealed an operon whose gene products were homologous to multidrug efflux systems found in a variety of Gram-negative bacteria [35]. These include a membrane fusion protein (*amrA*), an RND-type transporter (*amrB*), an outer membrane protein (*oprA*), and a divergently transcribed regulator protein (*amrR*). We have given the genes involved in aminoglycoside and macrolide resistance in *B. pseudomallei* the prefix *amr* (aminoglycoside, macrolide resistance) and refer to the operon-encoded proteins as AmrAB-OprA. Consistent with the presence of an efflux system specific for aminoglycosides, both mutants accumulated [^3H]streptomycin, whereas the parent strain did not. Regu-

lator (*amrR*) knockout mutants demonstrated increased aminoglycoside minimum inhibitory concentrations (MICs), suggesting that the regulator protein serves as a transcriptional repressor of the efflux operon. While efflux-mediated resistance to macrolide antibiotics has been reported in bacteria [36-38], to our knowledge, the AmrAB-OprA efflux system is a unique mechanism for high-level aminoglycoside resistance. A hallmark of Gram-negative multidrug efflux systems is energy-dependent drug exclusion [35]. Thus, collapse of a proton gradient across the cell membrane by addition of carbonylcyanide-m-chlorophenylhydrazone (CCCP) should result in a marked increase in drug accumulation. Surprisingly, efflux of streptomycin was not inhibited in *B. pseudomallei* by the addition of CCCP. However, we have recently sequenced a region of the *B. pseudomallei* chromosome that encodes a product with strong homology to *emrA* from *E. coli* [39], a locus reported to confer resistance to CCCP. We are currently examining this homolog in *B. pseudomallei* to determine if this may account for the inability of CCCP to inhibit aminoglycoside efflux.

B. pseudomallei also demonstrates high intrinsic resistance to the action of cationic antimicrobial peptides including human neutrophil peptides, polymyxins, protamine sulfate, melittin, magainins, and poly-L-lysine. In susceptible Gram-negative bacteria, cationic antimicrobial agents permeabilize the inner and outer membranes, resulting in cell death. As a model for understanding resistance to cationic peptides in the human host, we have been examining polymyxin B resistance in *B. pseudomallei*. MIC for polymyxin B for *B. pseudomallei* has been determined to be in excess of 128 000 $\mu\text{g/mL}$ (c.f. *E. coli* = .02-11.1 $\mu\text{g/mL}$ [40]), and MICs for poly-L-lysine and protamine sulfate exceed 20 $\mu\text{g/mL}$. Three Tn5-OT182 mutants have been isolated that show a greater than 500-fold increased susceptibility to the action of polymyxin B, with MICs ranging from 32-256 $\mu\text{g/mL}$. These mutants are also susceptible to the action of colistin (polymyxin E), but are still resistant to protamine sulfate and poly-L-lysine. Two of these mutants, PMB7 and PMB20, contain transposon integrations in genes involved in LPS core synthesis and include a *rfaF* gene (*waaF*) homolog encoding a putative heptosyl transferase and a gene whose product encodes a putative UDP-glucose dehydrogenase. These mutants demonstrate altered lipopolysaccharide and outer membrane profiles as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The transposon insertion in the third mutant, PMB4, is in a locus whose product shows homology to the *lytB* gene product of *E. coli*. In *E. coli* *lytB* regulates *relA*, which is involved in the stringent response [41]. This mutant also has an altered outer membrane profile. Characterization of these three loci and their products should provide us with a more thorough understanding of polymyxin B resistance in *B. pseudomallei*.

9. Vaccines

While melioidosis is becoming increasingly recognized as a significant cause of morbidity and mortality in endemic regions, there is currently no licensed vaccine

available for immunoprophylaxis against this disease. To this end, our laboratory has devoted a considerable amount of attention towards identifying and characterizing antigens expressed by *B. pseudomallei* isolates for the intended purpose of synthesizing vaccine candidates to immunize high-risk populations against melioidosis. In order to accomplish this we have routinely employed a combination of molecular, biochemical, and immunological techniques. We have been quite successful at identifying a variety of secreted and cell-associated antigens via this approach (DeShazer, unpublished) [13]. Evidence from our studies now strongly suggests that the most promising candidates for the rational design of an active vaccine construct are the flagellin protein and the O-polysaccharide (PS) moieties derived from endotoxin expressed by *B. pseudomallei* isolates. To support this hypothesis, we have successfully demonstrated that both polyclonal and monoclonal antisera raised against *B. pseudomallei* flagellin proteins, lipopolysaccharide, and a tetanus toxin-PS glycoconjugate are able to provide passive protection against bacterial challenge using the diabetic infant rat infection model [42, 43]. Similarly, serological and spectroscopic analysis of *B. pseudomallei* flagellin proteins and PS moieties in coordination with the molecular characterization of the genetic loci which encode for these factors has also confirmed that these antigens are remarkably conserved both structurally and antigenically throughout the species [13, 26, 44]. In fact, there is mounting evidence to suggest that there may actually be only one *B. pseudomallei* serotype which is representative of the species in general [45]. Therefore, based upon these observations and considering the options available to us, we have concluded that a conjugate molecule incorporating both flagellin and PS is a reasonable vaccine candidate for actively immunizing against melioidosis.

The flagellin-PS conjugate approach certainly possesses a number of desirable attributes that need be considered in the rational design of a vaccine candidate. Firstly, the incorporation of two protective antigens from the same organism enhances the immunological repertoire of the vaccine recipient, while concomitantly affording protection against strains that may become antigenically varied with respect to one of the two components via spontaneous mutation or gene recombination events. The conjugation of the PS to the flagellin carrier has also enabled us to augment immune responses against the T-cell-independent type-2 PS component while simultaneously evoking desirable immunoglobulin class switching events. Most importantly, the use of an active vaccine containing only the PS portion of LPS, but not the toxic lipid A component, conjugated to the flagellin carrier has obviated the potential for toxic side effects associated with intact LPS. This molecule does, however, take advantage of the protective capacities inherent to the PS moiety. Using this approach, we have immunized rabbits with a flagellin-PS conjugate which incorporates adipic acid dihydrazide as a spacer, and we have been able to elicit high-titer immunoglobulin G responses to both the carbohydrate and protein components of the construct [46]. Furthermore, the IgG fraction of the immune serum was capable of protecting diabetic rats from a *B. pseudomallei*

challenge. In these studies, the 50% lethal dose value calculated for the control group was 7.5×10^3 CFU, while that for the immunized group was calculated to be 8.9×10^5 CFU. Due to the success of these preliminary studies, we are currently testing the efficacy of this vaccine preparation in active immunization trials.

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Antibiotic resistance mechanisms of *Burkholderia pseudomallei*

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Introduction

Burkholderia pseudomallei is a common cause of human pneumonia and fatal bacteremias in endemic areas (1). The organism is an opportunistic pathogen, and those individuals particularly susceptible to *B. pseudomallei* infection have underlying conditions such as diabetes or renal disease (1,2). Clinical manifestations of *B. pseudomallei* infection, a disease known as melioidosis, vary greatly from an asymptomatic state, to benign pneumonitis, to acute or chronic pneumonia or to overwhelming septicemia (3). Treatment of melioidosis can involve up to 9 months of antibiotic

therapy (4), and relapse of disease is common. Additionally, the latency period of the organism may vary from 2 days to 26 years (3,5).

Prior to the use of antimicrobials, the mortality of apparent *B. pseudomallei* infections was 95%. French experience in Indochina indicated that with chloramphenicol therapy the mortality was 20%. With better diagnosis and more prolonged appropriate therapy, mortality in forms except the septicemic should be low (1,6). Even with vigorous appropriate antibiotics and supportive therapy, the mortality in patients with melioidosis septicemia is 40% or greater. Successfully treating melioidosis patients is difficult because *B. pseudomallei* is inherently resistant to a variety of antibiotics including β -lactams, aminoglycosides, macrolides

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and polymyxins (7,8).

Resistance to Aminoglycosides

We have utilized Tn5-OT182 mutagenesis to identify *B. pseudomallei* genes involved in aminoglycoside (9) resistance. Initial experiments designed to identify genes involved in aminoglycoside resistance resulted in the isolation of two transposon mutants, *B. pseudomallei* RM101 and RM102, which had an 8-128 fold increase in susceptibility to the aminoglycosides streptomycin, gentamicin, neomycin, tobramycin, kanamycin and spectinomycin. Further examination revealed that the mutants, in contrast to the parent strain, were also susceptible to the macrolides erythromycin and clarithromycin but not to the lincosamide clindamycin. Sequencing of DNA flanking the transposon insertions revealed an operon whose gene products were homologous to antibiotic efflux systems found in a variety of gram-negative bacteria (10). These gene products include a membrane fusion protein (*amrA*), a RND-type transporter (*amrB*), an outer membrane protein (*oprA*) and a divergently transcribed regulator protein (*amrR*). We have given the genes involved in aminoglycoside and macrolide resistance in *B. pseudomallei* the prefix *amr* (aminoglycoside, macrolide resistance) and refer to the operon as AmrAB-OprA.

Consistent with the presence of an efflux system specific for aminoglycosides,

both mutants accumulated ^3H -streptomycin, whereas the parent strain did not. While efflux mediated resistance to macrolide antibiotics has been reported in bacteria (11-13), to our knowledge, the AmrAB-OprA efflux system is a unique mechanism for high-level aminoglycoside resistance. A hallmark of gram-negative multidrug efflux systems is energy-dependent drug exclusion (10). Thus, collapse of a proton gradient across the cell membrane by addition of carbonylcyanide-*m*-chlorophenylhydrazone (CCCP) should result in a marked increase of drug accumulation. Surprisingly, efflux of streptomycin was not inhibited in *B. pseudomallei* by the addition of CCCP. However, we have recently sequenced a region of the *B. pseudomallei* chromosome that encodes proteins that may be involved in resistance to hydrophobic chemicals and antibiotics. This locus contains three open reading frames which we have named OrfE, D and F. The OrfE gene product is homologous to MarR from *E. coli* and serves as a repressor for the multiple antibiotic resistance operon, *marRAB* (14). The Orf D gene encodes a protein with homology to outer membrane proteins OpcM from *B. cepacia* (15), and OprM, K and N from *P. aeruginosa* (16). The last gene in this locus, OrfF,

encodes a protein with homology to the EmrB protein of *E. coli*. This membrane protein belongs to the major facilitator family of translocases and confers resistance to highly hydrophobic substances including CCCP (17). We are currently examining this locus in *B. pseudomallei* to determine its role in resistance to CCCP and hydrophobic antibiotics. Characterization of these gene products and their functions will contribute to the understanding of inherent antibiotic resistance in this organism.

Resistance to Cationic Peptides

Burkholderia pseudomallei is resistant to the killing action of a variety of cationic antimicrobial substances including human neutrophil peptide (HNP-1), protamine sulfate, poly-L-lysine, magainins and polymyxins. In susceptible Gram negative bacteria, cationic antimicrobial agents effectively permeabilize the outer and inner membranes leading to cell death. Recent studies have shown that *B. pseudomallei* 1026b can survive in media containing >100 mg/ml of polymyxin B (PMB) and polymyxin E (colistin) (18) compared to *E. coli* cells that are killed at very low concentrations (MIC 0.2 – 11.1 µg/ml). In addition, this organism is resistant to the cationic peptides protamine sulfate

(PS) and poly-L-lysine at concentrations > 20 mg/ml. PMB susceptible mutants of *B. pseudomallei* 1026b have been isolated using Tn5-OT182 mutagenesis, these mutants, however, remain resistant to PS and poly-L-lysine. The PMB susceptible mutants can be divided into two groups, one group possessing altered LPS and changes in outer membrane proteins, and the other group showing only outer membrane protein changes (18). The LPS mutants have disruptions in gene homologs involved in core oligosaccharide biosynthesis and these mutants demonstrate the loss of both the O-polysaccharide moiety and the outer core region of LPS Type II. The latter group of mutants harbor Tn5-OT182 integrations in a *lytB* gene homolog. The product of this gene has been shown to affect peptidoglycan and phospholipid biosynthesis in other Gram negative bacteria (19), therefore it is reasonable to suggest that the envelope of these mutants may be severely compromised. Together these studies indicate that it is the architecture of the *B. pseudomallei* cell that provides an effective permeability barrier against the action of PMB. Current studies are underway in order to define the mechanism(s) of resistance against the action of PS, poly-L-lysine and HNP-1. It is anticipated that these studies will contribute to our understanding of the mechanism(s) used by this organism to resist the killing action of cationic

antimicrobial substances found in host immune cells.

Resistance to β -Lactams

B. pseudomallei is naturally resistant to many penicillins and early generation-cephalosporins (7,20-22). β -lactam resistance has been linked to the production of an inducible chromosomal β -lactamase (23,24). Based on the similarity of this β -lactamase to one from *Proteus vulgaris*, e.g. its size and hydrolytic profile, this enzyme is believed to be an oxyimino-cephalosporinase and is placed into the Richmond and Sykes class 1, group 1c, or group 2e by 1995 Bush-Jacoby-Medeiros classification (23,25). Because it is susceptible to inhibition by β -lactamase inhibitors, such as clavulanic acid, it is most likely that the *B. pseudomallei* β -lactamase belongs to the class A, rather than the class C enzymes. Further, in those cases where amino acid sequences are available, all chromosomal β -lactamases in Bush group 2e, belong to the class A β -lactamases (26-32). Recently, a chromosomal class A β -lactamase has been reported in *B. cepacia* 249 (32). Still, there are no sequence data available for *B. pseudomallei*, and further study is needed to find the exact molecular class of its enzyme.

The β -lactamase from *B. pseudomallei* has a high V_{\max}/K_m for carbenicillin, cephaloridine, cephalothin, cefuroxime, and cefotaxime that correlates rather well with their MICs (24). The extended-spectrum β -lactams, such as ceftazidime, imipenem or the combination of amoxicillin/clavulanic acid may be used successfully in the treatment of melioidosis (33-37). However, as in many Gram negative bacteria, the selective pressure from antibiotic treatment sometimes causes this organism to develop additional resistance against ceftazidime or amoxicillin/clavulanic acid (20). The genetic control of this emerging resistance has not been extensively studied, but mutations leading to constitutive production of chromosomal β -lactamase and/or changes in substrate hydrolytic profile has been demonstrated (23). It is noteworthy that certain changes in critical amino acid residues around the active site pocket of a β -lactamase can be selected by the selective pressure from the use of antibiotics (38-41). The horizontal acquisition of the gene encoding for extended-spectrum β -lactamase has never been reported in *B. pseudomallei*. Attempts to find plasmids in resistant clinical isolates have been unsuccessful (23); however, the possibility of horizontal gene transfer in *B. pseudomallei* cannot be discounted,

especially when the pool of resistance genes now are much higher than in the past.

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Pathogenesis of Melioidosis: Use of Tn5-OT182 to Study the Molecular Basis of *Burkholderia pseudomallei* Virulence

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Abstract

Burkholderia pseudomallei is a human and animal pathogen in tropical regions, especially Southeast Asia and northern Australia. In this review, we summarize recent data on the use of the transposon Tn5-OT182 to identify and characterize putative virulence determinants of *B. pseudomallei*. Tn5-OT182 is a self-cloning, promoter probe transposon that integrates randomly in the *B. pseudomallei* genome. Tn5-OT182 mutants deficient in motility, serum resistance, invasion, secretion of exoproducts and aminoglycoside resistance are described and the genes encoding each of these phenotypic traits are analyzed. The Tn5-OT182 mutants are compared with the parental strain in two animal models of *B. pseudomallei* infection in order to assess the relative importance of the mutated genes in virulence. The data presented in this review demonstrate that Tn5-OT182 is a useful transposon for the genetic analysis of putative virulence determinants of *B. pseudomallei*. (*J Infect Dis Antimicrob Agents* 1999;16:91-6.)

INTRODUCTION

Melioidosis, an infection of humans and animals, is caused by the Gram-negative bacterium *Burkholderia pseudomallei*.¹⁻⁵ The disease is most common in Southeast Asia and northern Australia, but sporadic cases have also been described in other regions of the world. The organism can be isolated from wet soil in endemic regions.⁶⁻⁸ The route of infection is probably via inhalation of dust particles or direct inoculation of contaminated soil into cuts or abrasions. The outcome of a *B. pseudomallei* infection can vary from asymptomatic seroconversion to fulminant septicemic melioidosis and death. Acute septicemic melioidosis is the most severe manifestation of *B. pseudomallei* infection. The majority of culture-positive melioidosis patients are bacteremic upon admission to the hospital, and many are clinically septicemic.^{5,9} Even with aggressive antimicrobial therapy, the mortality rate

of acute septicemic melioidosis is approximately 40 percent.¹⁰ Acute or chronic infection of any organ can occur, and lesions can form on any tissue but are most commonly found in the lungs, liver, spleen, lymph nodes, skin and soft tissues and urinary tract.⁵ Latent infections can also occur in which the organism can lay dormant for as many as 26 years before recrudescence into an active infection.^{11,12} *B. pseudomallei* appears to be an opportunistic pathogen as a relatively high number of melioidosis patients have underlying diseases such as diabetes mellitus and renal failure.¹³⁻¹⁷

B. pseudomallei produces a number of factors that may be involved in pathogenesis. These include secreted products such as protease, lipase, lecithinase and a heat-labile toxin as well as cell-associated products such as lipopolysaccharide (LPS), flagella, exopolysaccharide (EPS) and pili.¹⁸⁻²⁴ The outcome of the host-pathogen interaction during infection may

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also be influenced by the ability of *B. pseudomallei* to resist killing by normal human serum (NHS),^{25,26} survive within phagocytic cells and invade nonphagocytic cells.^{27,28}

We are interested in identifying and characterizing those factors that are responsible for *B. pseudomallei* pathogenesis at the molecular level. We have previously described the use of two animal models of acute *B. pseudomallei* infection, diabetic rats²⁹ and Syrian hamsters.³⁰ The relative virulence of *B. pseudomallei* mutants can be determined using these animal models of melioidosis, and the importance of the mutated genes in pathogenesis can be ascertained. In this review, we will summarize recent data on the use of Tn5-OT182 for the identification and characterization of putative virulence factors of *B. pseudomallei*.

Tn5-OT182 mutagenesis

The transposon described here is a Tn5-derivative termed Tn5-OT182 (Fig. 1).³¹ Tn5 is well characterized genetically, and it transposes with relatively high frequencies in many Gram-negative bacteria and generally integrates with little sequence specificity.³² Tn5-OT182 contains a tetracycline resistance determinant which is a useful selective marker in *B. pseudomallei*. In addition, this transposon contains the pBR325 origin of replication which allows the "self-cloning" of DNA immediately flanking the site of Tn5-OT182 integration.^{31,33} Finally, the presence of a promoter-less *lacZ* reporter gene on this transposon allows the formation of *lacZ* transcriptional fusions when Tn5-OT182 integrates downstream of a functional promoter (Fig. 1).

We found that Tn5-OT182 mutagenesis of *B. pseudomallei* was strain-dependent.³³ The inability of some *B. pseudomallei* strains to be mutagenized by Tn5-OT182 may be due to inefficient transfer of the Tn5-OT182 carrier plasmid, pOT182, to recipient cells as a result of restriction systems, surface exclusion phenomena or lack of essential envelope functions.³⁴ In addition, the efficiency of Tn5-OT182 mutagenesis can vary as much as ten-fold between different *B. pseudomallei* strains.³³ We found that mutagenesis was most efficient with *B. pseudomallei* 1026b, a strain isolated in Thailand from a human case of septicemic melioidosis with skin, soft tissue and spleen involvement. It has all the typical phenotypic characteristics of *B. pseudomallei*³⁰ and is highly virulent in both Syrian hamsters and infant diabetic rats (Table 1). In the following sections, we will describe Tn5-OT182 mutants of *B. pseudomallei* 1026b that are deficient in the production of putative virulence determinants.

Motility mutants

We have previously shown that polyclonal anti-flagellin antisera obtained from rabbits immunized with purified flagellin passively protected diabetic rats from an intraperitoneal (i.p.) challenge with *B. pseudomallei*.¹⁹ Our interpretation of this result is that motility and/or the presence of a flagellum may be an important virulence determinant of this organism and that flagellin may serve as a protective antigen. In order to determine the relative importance of motility in virulence, we isolated and characterized *B. pseudomallei* motility mutants.

We screened approximately 3,500 Tn5-OT182

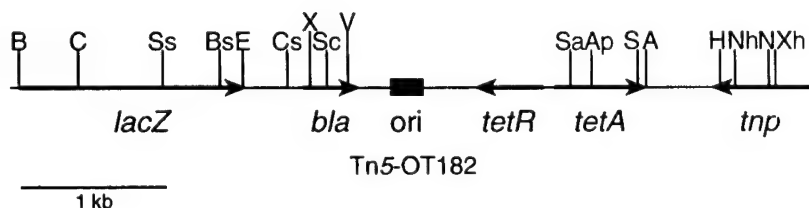


Fig. 1 Physical and genetic map of Tn5-OT182. The arrows represent the location and direction of transcription of genes. The black box represents the pBR325 origin of replication. *lacZ*, promoter less gene encoding β -galactosidase; *bla*, β -lactamase; *ori*, pBR325 origin of replication; *tetR*, tetracycline resistance genes from RP4; *tnp*, transposase; B, *Bam*HI; C, *Cla*I; Ss, *Sst*I; Bs, *Bsi*WI; E, *Eco*RI; Cs, *Csp*45I; X, *Xmn*I; Sc, *Sca*I; V, *Vsp*I; Sa, *Sal*I; Ap, *Apa*I; S, *Stu*I; A, *Avr*II; H, *Hind*III; Nh, *Nhe*I; N, *Not*I; and Xh, *Xho*I. Tn5-OT182 is 10,705 bp in length, and the entire nucleotide sequence is in the Genbank database under the accession number U73849.

Table 1. Relative virulence of *B. pseudomallei* strains in Syrian hamsters and infant diabetic rats.

Strain	Relevant characteristics	LD ₅₀ ^a	
		Syrian hamster ^b	Infant diabetic rat ^c
1026b	Clinical isolate	< 5	2 x 10 ⁴
MM36	Motility mutant; <i>fliC</i> ::Tn5-OT182	8	8 x 10 ³
SRM117	Serum sensitive mutant; <i>wbiL</i> ::Tn5-OT182	62	> 5 x 10 ⁶
AJID8	Invasion-deficient mutant; <i>irIR</i> ::Tn5-OT182	< 10	2 x 10 ⁴
DD213	Secretion mutant; <i>gspD</i> ::Tn5-OT182	13	ND
RM101	Aminoglycoside and macrolide sensitive mutant; <i>amrA</i> ::Tn5-OT182	< 10	ND

^a, 50 percent lethal dose^b, 2 days^c, 7 days

ND, not determined

mutants for their motility phenotype on motility agar plates, and 28 non-motile mutants were identified.³³ The DNA flanking Tn5-OT182 in each motility mutant was isolated by self-cloning, and the nucleotide sequence was determined.^{31,33} The nucleotide sequences obtained from 22 of the 28 motility mutants predicted products that contained homologous proteins in the nonredundant sequence database. Most of the homologous proteins identified were involved in flagellin synthesis and operation in *E. coli* and *S. typhimurium*. These include chemotaxis proteins (Tsr, CheA and CheW), switch proteins (FliM and FliN), flagellar export proteins (FliR, FliP, FliQ, and FliH), flagellar assembly proteins (FliB and FliE), a rod protein (FlgB), the L-ring protein (FlgH), the hook protein (FlgE), a hook-associated protein (FlgL), a hook scaffolding protein (FlgD), a protein involved in regulation of hook length (FliK), a flagellin motor protein (MotA), and the flagellar subunit protein (FliC).³⁵ We conclude that synthesis and operation of the flagellar apparatus in *B. pseudomallei* requires multiple genes that presumably encode proteins with similar functions to those found in *E. coli* and *S. typhimurium*. The fact that such a variety of motility genes were identified by this mutagenesis procedure demonstrates the random nature of integration of Tn5-OT182 in the *B. pseudomallei* 1026b chromosome. Furthermore, we demonstrated that the promoter-less *lacZ* of Tn5-OT182 functions as an efficient promoter probe in *B. pseudomallei*.³³

The strains MM35 and MM36 contained Tn5-OT182 integrations in the flagellin structural gene, *fliC*. We found no significant difference in the virulence of MM36 and 1026b in infant diabetic rats or

Syrian hamsters (Table 1). On the other hand, we previously demonstrated that polyclonal rabbit antisera raised against purified flagellin passively protected diabetic rats from challenge with *B. pseudomallei*.¹⁹ Taken together, these results indicate that while flagella and/or motility are probably not virulence determinants in these animal models of infection, purified flagellin may still serve as a protective immunogen against *B. pseudomallei* infection.

Serum sensitive mutants

The ability of *B. pseudomallei* to invade and survive within the bloodstream is clearly important in the pathogenesis of septicemic melioidosis. *B. pseudomallei* is highly resistant to the bactericidal activity of NHS and may avoid immune elimination by its ability to resist complement-mediated bacteriolysis.^{25,26} The ability to evade complement-mediated killing is considered to be an important virulence determinant of invasive Gram-negative bacteria.^{36,37}

We have found that *B. pseudomallei* 1026b multiplies in 10-30 percent NHS, and we developed a simple screen for the identification of serum sensitive mutants based on this novel phenotype (DeShazer D, Brett PJ and Woods DE, unpublished). Approximately 1200 Tn5-OT182 mutants were screened, and three serum sensitive mutants were identified. The type II O-antigenic polysaccharide (O-PS) moiety of LPS²³ was absent in the serum sensitive mutants, but the type I O-PS moiety of LPS and EPS were present. A representative serum sensitive mutant, SRM117, was killed by the alternative pathway of complement. The Tn5-OT182 integrations in the

serum sensitive mutants were physically linked on the *B. pseudomallei* chromosome, and further genetic analysis of this locus revealed a cluster of 15 genes required for the biosynthesis of type II O-PS and serum resistance. The products encoded by the type II O-PS biosynthetic gene cluster were similar to proteins involved in polysaccharide biosynthesis in other bacteria.

The serum sensitive mutant SRM117 contains a Tn5-OT182 integration in the last gene of the type II O-PS biosynthetic gene cluster (*wbiI*). SRM117 was compared to the parental strain 1026b in two animal models of *B. pseudomallei* infection: hamsters and infant diabetic rats (Table 1). SRM117 was approximately 10-fold less virulent than 1026b in the hamster model of melioidosis and greater than 100-fold less virulent than 1026b in the infant diabetic rat model of melioidosis. These results indicate that the type II O-PS moiety of LPS is essential for *B. pseudomallei* serum resistance and virulence.

Invasion-deficient mutant

B. pseudomallei is a facultative intracellular pathogen capable of invasion of non-professional phagocytes *in vitro*.^{27,28} The relative importance of invasion in the pathogenesis of melioidosis is currently unknown. The interaction of *B. pseudomallei* with A549 cells, an established pulmonary epithelial cell line, provides a means to study the early phase of human respiratory tract infection by this organism. Tn5-OT182 mutants of *B. pseudomallei* 1026b were screened for their ability to invade A549 cells.²⁸ Approximately 1000 Tn5-OT182 mutants were screened, and one invasion-deficient mutant, AJ1D8, was identified. AJ1D8 exhibited invasion levels that were 11 percent or less than the invasion level of 1026b. In addition, the inability of AJ1D8 to invade eukaryotic cells appeared to be a general phenomenon and was not specific to A549 cells. There was no difference in the ability of 1026b and AJ1D8 to resist killing by RAW macrophages or the human defensin HNP-1.

The nucleotide sequence flanking the Tn5-OT182 integration in AJ1D8 was determined, and two open reading frames were identified. The predicted proteins shared considerable homology with two-component regulatory systems involved in the regulation of heavy metal resistance in other organisms. AJ1D8 was 16-fold more sensitive to Cd²⁺ and two-fold more sensitive to Zn²⁺ than 1026b but was not sensitive to any of the other heavy metals examined. The *B. pseudo-*

mallei two-component regulatory system, termed *irlRS*, complemented the invasion-deficient and heavy metal sensitive phenotype of AJ1D8 *in trans*.²⁸

The virulence of the invasion-deficient mutant, AJ1D8, was assessed in Syrian hamsters and diabetic infant rats (Table 1). There was no significant difference in the virulence of AJ1D8 as compared to 1026b in Syrian hamsters or infant diabetic rats, suggesting that the *irlRS* locus is probably not a virulence determinant in these acute animal models of *B. pseudomallei* infection.²⁸

Secretion mutants

B. pseudomallei secretes several potential virulence factors including protease, lipase and lecithinase.¹⁸ The genes for these factors have not been identified, but the mechanism of secretion has recently been elucidated (DeShazer D, Brett PJ, Burtnick M and Woods DE, unpublished). Twenty-nine unique Tn5-OT182 mutants unable to secrete protease, lipase or lecithinase were identified on 3 percent skim milk plates and egg yolk agar plates. The Tn5-OT182 integrations in these mutants were mapped to two distinct loci. Twenty-six of the mutations were clustered at a genetic locus spanning approximately 12 kb. The DNA sequence of this region consists of 10 genes, *gspDEFGHIJKLM*, which encode proteins with a high degree of homology to general secretory or type II secretion pathway proteins from a variety of Gram-negative bacteria.³⁸ In addition, three mutations were identified within an unlinked gene (*gspO*) which encoded a protein with homology to type IV prepilin peptidases. The total amount of protease produced by 1026b and DD213, a representative secretion mutant, was similar. The majority of the protease produced by 1026b was found in the supernatant. In contrast, the majority of the protease produced by DD213 was found in the cell-associated fraction. Taken together, the results of these studies suggest that protease, lipase and lecithinase are secreted by a type II secretion pathway.

The relative virulence of the secretion mutant DD213 was assessed in Syrian hamsters (Table 1). We found that there was only a small difference in the virulence of DD213 and 1026b in Syrian hamsters. The relative virulence of DD213 in the infant diabetic rat model of *B. pseudomallei* infection needs to be determined before the importance of secreted products in the pathogenesis of melioidosis can be assessed. Furthermore, mutants deficient only in protease, lipase or lecithinase are necessary for analyzing the signifi-

cance of these exoproducts as virulence determinants.

Aminoglycoside and macrolide sensitive mutants

B. pseudomallei is intrinsically resistant to a wide range of antimicrobial agents including β -lactams, aminoglycosides, macrolides and polymyxins.³⁹ While antibiotic resistance is not a *B. pseudomallei* virulence determinant, it is important clinically as most parts of the world commonly use a combination of penicillin and gentamicin as an initial treatment of community-acquired septicemia.¹⁷ Penicillin and gentamicin are ineffective against *B. pseudomallei*, a major cause of community-acquired septicemia in northeast Thailand.¹⁷

We used Tn5-OT182 to mutagenize *B. pseudomallei* 1026b to identify genes involved in aminoglycoside resistance (Moore RA, DeShazer D, Reckseidler S, Weissman A and Woods DE, unpublished). Approximately 3,000 transposon mutants were screened for susceptibility to streptomycin and tobramycin. Two mutants, RM101 and RM102, were isolated which had an 8-128 fold increase in susceptibility to the aminoglycosides streptomycin, gentamicin, neomycin, tobramycin, kanamycin and spectinomycin. In addition, both mutants, in contrast to the parental strain, were susceptible to the macrolides erythromycin and clarithromycin but not to the lincosamide clindamycin. The Tn5-OT182 integrations in RM101 and RM102 were mapped to a three gene operon, *amrA*, *amrB* and *oprA*, which encoded products with homology to a RND-type transporter, a membrane fusion protein and an outer membrane protein, respectively. These proteins are components of multidrug efflux systems that use the energy of the proton motive force to extrude drugs from the cell.^{40,41} Upstream of the three gene operon was a divergently transcribed gene, *amrR*, that encoded a product with homology to proteins involved in transcriptional regulation of multidrug efflux systems. Consistent with the presence of an active efflux system, RM101 and RM102 accumulated ³H-streptomycin, whereas 1026b did not. Furthermore, an *amrR* null mutant had increased aminoglycoside minimal inhibitory concentrations suggesting that the protein encoded by this gene serves as a transcriptional repressor of the efflux operon. These results indicate that aminoglycoside and macrolide resistance in *B. pseudomallei* is due to an active efflux system that extrudes these drugs from the cell. As expected, we found no significant difference in the virulence of RM101 and 1026b in Syrian hamsters (Table 1).

DISCUSSION

Tn5-OT182 has several useful features that make it an excellent transposon for use in *B. pseudomallei*. Tn5-OT182 integrates randomly into the *B. pseudomallei* genome, and most non-lethal genes can be identified at a reasonable frequency. In general, if an appropriate screen exists for the gain or loss of a particular phenotypic trait, the gene or genes encoding this phenotype can be identified using Tn5-OT182. The tetracycline resistance determinant of Tn5-OT182, *tetA*, is expressed in *B. pseudomallei* and serves as an efficient selective marker in this organism.³³ Since *B. pseudomallei* does not produce β -galactosidase,⁴² the promoterless *lacZ* of Tn5-OT182 functions as a promoter probe in this organism, allowing the analysis of transcription of a mutated gene. The most useful feature of Tn5-OT182, however, is the ability to self-clone the DNA immediately flanking the site of integration.³¹ The nucleotide sequence flanking the Tn5-OT182 integration can then be directly sequenced with conserved oligonucleotide primers that bind to the ends of the transposon.³³

In this review, we have described the use of Tn5-OT182 mutagenesis to study potential virulence determinants of *B. pseudomallei*. We have identified and characterized Tn5-OT182 mutants deficient in motility, serum resistance, invasion and secretion of exoproducts. The relative virulence of representative mutants were assessed in the infant diabetic rat and/or the Syrian hamster model of *B. pseudomallei* infection (Table 1). The most important virulence determinant that we have identified using Tn5-OT182 mutagenesis and an animal model of infection is the type II O-PS moiety of LPS, which confers serum resistance by preventing killing of *B. pseudomallei* by the alternative pathway of complement. Motility, invasion and secretion of exoproducts do not appear to be critical for *B. pseudomallei* virulence in Syrian hamsters and/or infant diabetic rats. Finally, we have described aminoglycoside and macrolide sensitive mutants of *B. pseudomallei*. The Tn5-OT182-tagged genes in these mutants encode a novel multidrug efflux pump that extrudes aminoglycosides and macrolides out of the cell and away from their targets.

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NOTES

Molecular Characterization of Genetic Loci Required for Secretion of Exoproducts in *Burkholderia pseudomallei*

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Previous studies have demonstrated that *Burkholderia pseudomallei* secretes protease, lipase, and phospholipase C (PLC) into the extracellular milieu, but their mechanisms of secretion and roles in pathogenesis have not been elucidated. In this study, we isolated and characterized 29 transposon mutants unable to secrete protease, lipase, and PLC.

Melioidosis is an infection caused by the gram-negative bacillus *Burkholderia pseudomallei* (2, 15). In this report, we describe the isolation and characterization of *B. pseudomallei* 1026b transposon mutants deficient in secretion of protease, lipase, and phospholipase C (PLC). We also compared the relative virulence of 1026b and DD213, a representative secretion mutant, in the Syrian hamster model of melioidosis.

Isolation of secretion mutants of *B. pseudomallei* 1026b. The bacterial strains and plasmids used in this study are listed in Table 1. The goal of this study was to identify mutants unable to secrete protease, lipase, and PLC in order to further characterize the genes involved in this process and examine the relative importance of these exoproducts in the pathogenesis of melioidosis. We screened approximately 15,000 Tn5-OT182 (3) mutants of *B. pseudomallei* 1026b for the inability to form a zone of clearing around isolated colonies on 3% skim milk agar (protease activity). Twenty-nine distinct mutants that did not demonstrate a zone of clearing around isolated colonies were identified. In fact, none of the 29 mutants demonstrated a zone of clearing on tributyrin agar (lipase activity) or formed a cloudy (opaque) zone around areas of bacterial growth on egg yolk agar (PLC activity). Taken together, these results demonstrate that all 29 mutants were unable to secrete protease, lipase, and PLC. This strongly suggests that this organism utilizes the same secretion pathway for these exoproducts.

Protease and PLC are cell associated in *B. pseudomallei* DD213 and C21. We performed quantitative protease and PLC assays on the culture supernatants and cell lysates of 1026b and two representative secretion mutants, DD213 and C21 (Fig. 1). More than 98% of the 1026b proteolytic activity was present in the culture supernatant, indicating that protease is efficiently secreted by *B. pseudomallei* (Fig. 1A). In contrast, only 2% of the DD213 proteolytic activity and 7% of the C21 proteolytic activity were present in the culture supernatant (Fig. 1A). The total amounts of proteolytic activity (culture supernatant plus cell lysate) produced by 1026b, DD213, and C21 were similar (Fig. 1A). Approximately 53% of the 1026b PLC activity was

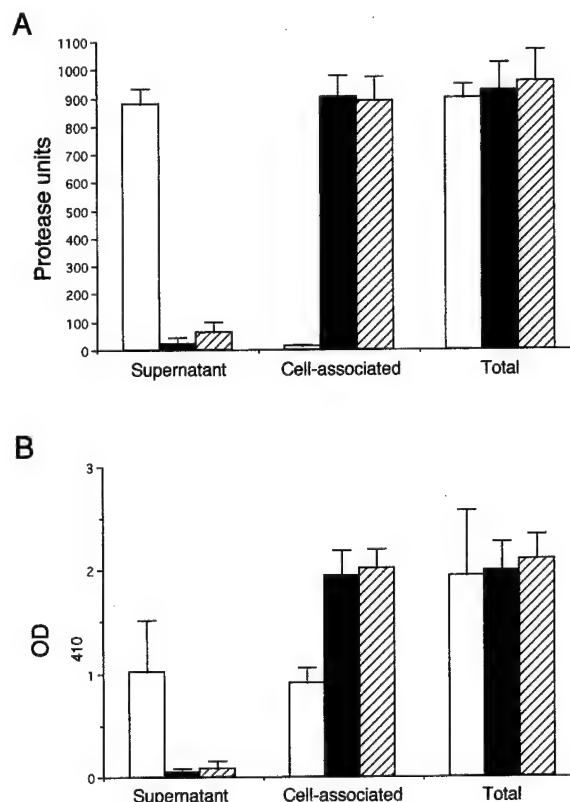


FIG. 1. Quantitative enzymatic assay of secreted (supernatant), cell associated, and total proteolytic and PLC activities of *B. pseudomallei* 1026b (□), DD213 (■), and C21 (▨). Bacterial strains were grown in Luria-Bertani broth for 24 h at 37°C with shaking (250 rpm). (A) Protease assay (11). Numerical values are presented as protease units per milliliter of culture. One unit of protease is the amount of enzyme which yields an increase in absorbance (at 595 nm) of 0.25/h at 37°C. (B) PLC assay (5). Nitrophenylphosphorylcholine hydrolysis by PLC was monitored spectrophotometrically by the measurement of *p*-nitrophenol at 410 nm. Total enzymatic activity is the sum of the secreted and cell-associated activities. All numerical values are the means of three separate experiments performed in triplicate plus standard deviations (error bars).

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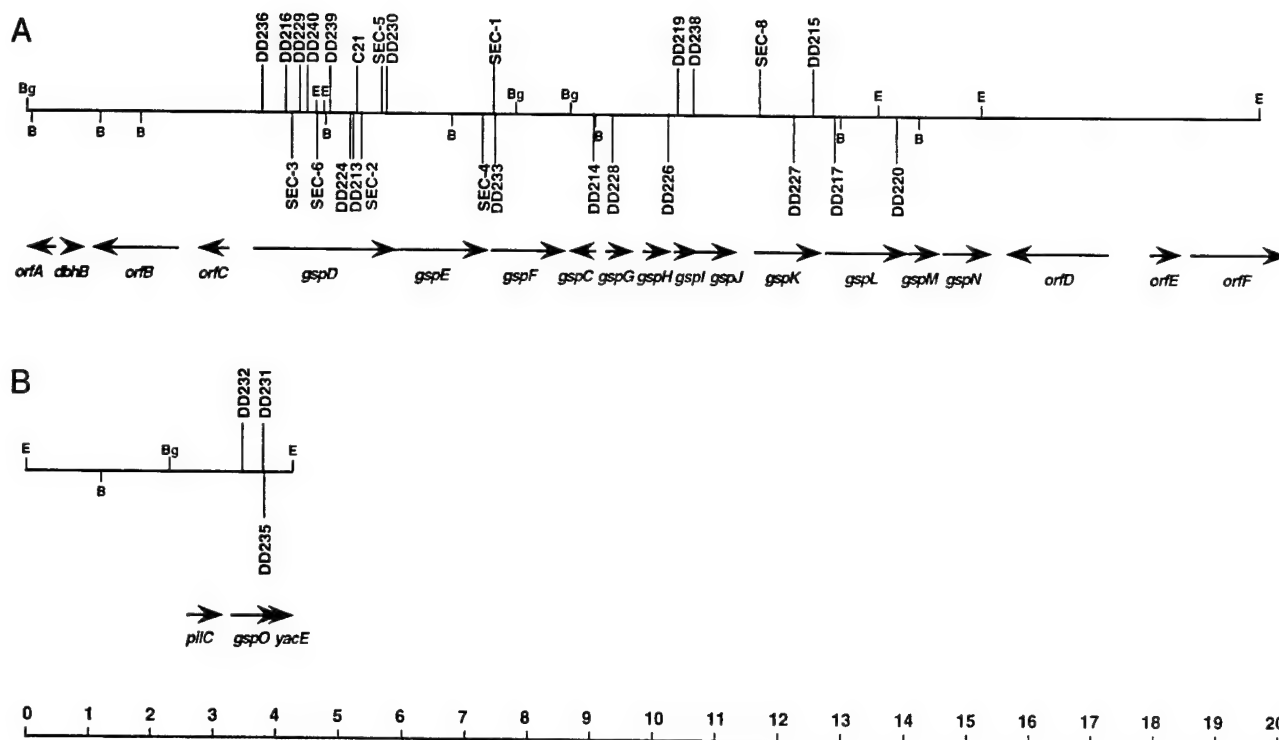


FIG. 2. Physical and genetic map of loci required for the secretion of protease, lipase, and PLC in *B. pseudomallei*. (A) Genetic locus containing 26 distinct Tn5-OT182 integrations in *B. pseudomallei* secretion mutants. (B) Genetic locus containing three distinct Tn5-OT182 integrations in *B. pseudomallei* secretion mutants. The horizontal lines represent *B. pseudomallei* chromosomal DNA, and the locations of relevant restriction endonuclease sites are shown (B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI). The exact locations of Tn5-OT182 integrations in the secretion mutants are shown by vertical lines extending above and below the chromosomal DNA. The promoterless *lacZ* gene of Tn5-OT182 integrations is oriented from left to right (5' to 3') in the secretion mutants designated by vertical lines extending above the chromosomal DNA and from right to left (5' to 3') in the secretion mutants designated by vertical lines extending below the chromosomal DNA. The locations and directions of the transcription of the genes are represented by arrows, and the gene names are shown below them. A scale (in kilobases) is shown at the bottom.

present in the culture supernatant, while the remaining 47% was cell associated (Fig. 1B). This result may indicate that PLC was inefficiently secreted under the assay conditions employed or that *B. pseudomallei* contains a cell-associated and a secreted PLC. DD213 and C21 culture supernatants contained only 2 and 3% of the total PLC activity, respectively (Fig. 1B). The total amounts of PLC activity produced by 1026b, DD213, and C21 were similar (Fig. 1B). These results clearly demonstrate that DD213 and C21 were secretion mutants; they produced normal levels of protease and PLC that accumulated

intracellularly rather than being secreted into the extracellular milieu.

Molecular characterization of the *B. pseudomallei* type II secretion pathway genes. The 29 *B. pseudomallei* secretion mutants isolated in this study contain Tn5-OT182 integrations in two distinct genetic loci (Fig. 2). The 26 Tn5-OT182 integrations presented in Fig. 2A were found within a gene cluster that encodes products that are homologous to type II secretion pathway proteins in gram-negative bacteria (10, 12). The genes in this cluster were named *gsp*, for "general secretory path-

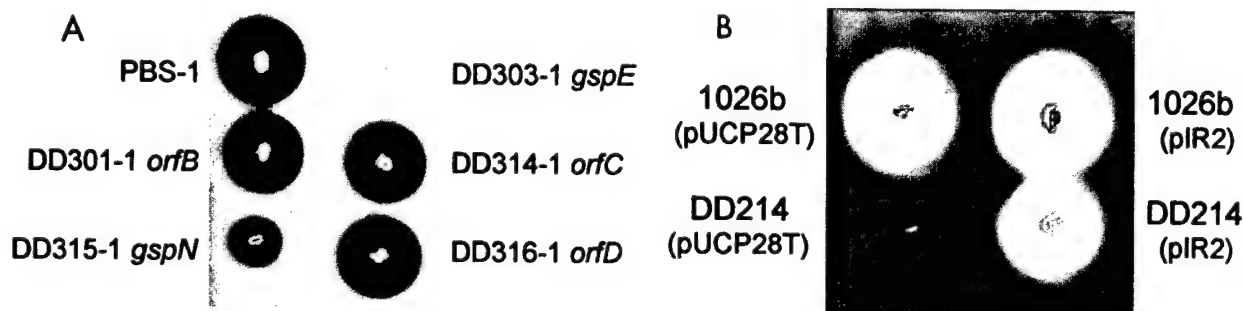


FIG. 3. Detection of protease secretion by *B. pseudomallei* strains on 3% skim milk agar. (A) Plasmid disruption of *gspE* and *gspN* resulted in strains that were completely or partially defective in protease secretion, respectively. The strains were grown on 3% skim milk agar plates containing tetracycline, and protease activity is indicated by a dark zone of clearing around the isolated colonies. (B) Complementation of the *gspC*::Tn5-OT182 mutation in DD214 by supplying *gspC* in trans on pIR2. The strains were grown on 3% skim milk agar plates containing trimethoprim, and protease activity is indicated by a light zone of clearing around the isolated colonies.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s) ^a	Reference or source
Strains		
<i>Escherichia coli</i>		
SM10	Mobilizing strain, transfer genes of RP4 integrated in chromosome; Km ^r Sm ^s	14
SM10 λ pir	SM10 with a λ prophage carrying the gene encoding the π protein	8
DH5 α	High-efficiency transformation	Bethesda Research Laboratories
SURE	High-efficiency transformation; deficient in homologous recombination; Km ^r Tc ^r	Stratagene
TOP10	General cloning and blue/white screening	Invitrogen
<i>B. pseudomallei</i>		
1026b	Clinical isolate; Sm ^r Tc ^s Tp ^s	3
DD303-1	1026b::pDD303-1; Tc ^r	This study
PBS-1	1026b::pPBS-1; Tc ^r	3
DD301-1	1026b::pDD301-1; Tc ^r	This study
DD314-1	1026b::pDD314-1; Tc ^r	This study
DD315-1	1026b::pDD315-1; Tc ^r	This study
DD316-1	1026b::pDD316-1; Tc ^r	This study
Plasmids		
pOT182	pSUP102(Gm)::Tn5-OT182; Cm ^r Gm ^r Ap ^r Tc ^r	7
pUCP28T	Broad-host-range vector; IncP OriT; pRO1600 ori; Tp ^r	13
pSKM11	Positive selection cloning vector; IncP OriT; ColE1 ori; Ap ^r Tc ^s	9
pC21Bg	18.5-kb <i>Bgl</i> II fragment from C21 obtained by self-cloning; Ap ^r Tc ^r	This study
pC21E	16.0-kb <i>Eco</i> RI fragment from C21 obtained by self-cloning; Ap ^r Tc ^r	This study
pDD226Nh	31.0-kb <i>Nhe</i> I fragment from DD226 obtained by self-cloning; Ap ^r Tc ^r	This study
pDD232E	8.4-kb <i>Eco</i> RI fragment from DD232 obtained by self-cloning; Ap ^r Tc ^r	This study
pDD235E	11.6-kb <i>Eco</i> RI fragment from DD235 obtained by self-cloning; Ap ^r Tc ^r	This study
pIR2	pUCP28T containing a 670-bp PCR product encompassing <i>gspC</i> ; Tp ^r	This study
pDD303-1	pSKM11 containing 750-bp <i>Xho</i> I- <i>Bam</i> HI fragment internal to <i>gspE</i> ; Ap ^r Tc ^r	This study
pPBS-1	pSKM11 containing 3' end of <i>fliC</i> and downstream DNA; Ap ^r Tc ^r	3
pDD301-1	pSKM11 containing 650-bp <i>Bam</i> HI fragment internal to <i>orfB</i> ; Ap ^r Tc ^r	This study
pDD314-1	pSKM11 containing a 390-bp PCR product internal to <i>orfC</i> ; Ap ^r Tc ^r	This study
pDD315-1	pSKM11 containing a 480-bp PCR product internal to <i>gspN</i> ; Ap ^r Tc ^r	This study
pDD316-1	pSKM11 containing 330-bp <i>Xho</i> I fragment internal to <i>orfD</i> ; Ap ^r Tc ^r	This study

^a ori, origin of replication.

way." The three Tn5-OT182 integrations presented in Fig. 2B are found within a gene (*gspO*) that encodes a product that is homologous to type IV prepilin peptidases (6) (Fig. 2B).

Identification of the 5' and 3' termini of the type II secretion pathway gene cluster. We constructed strains containing mutations in *orfB*, *orfC*, *gspN*, and *orfD* in order to investigate their roles in protein secretion (Fig. 2 and 3). The strains PBS-1 and DD303-1 were also constructed as positive and negative controls; respectively (Table 1). The zones of clearing produced by DD301-1 (*orfB*), DD314-1 (*orfC*), and DD316-1 (*orfD*) were indistinguishable from that produced by PBS-1 (Fig. 3A). Similar results were obtained with tributyrin agar and egg yolk agar (data not shown). The zone of clearing produced by DD315-1 (*gspN*) on 3% skim milk agar was consistently smaller than that produced by PBS-1 (Fig. 3A). Similar results were obtained with tributyrin agar and egg yolk agar (data not shown). The secretion phenotype of DD315-1 was probably not due to a polar effect because the gene immediately downstream of *gspN* (*orfD*) is not required for the secretion of exoproducts. These results demonstrate that *gspN* is required for efficient or maximal exoproduct secretion. The fact that we did not identify any Tn5-OT182 integrations in *gspN* supports the notion that mutations in this gene probably do not result in an absolute secretion-deficient phenotype. As *orfC* and *orfD* do not appear to be involved in the secretion of exoproteins by the type II pathway, we suggest that they define the 5' and 3' ends of the type II secretion pathway gene cluster, respectively (Fig. 2A).

***gspC* is essential for secretion of exoproducts in *B. pseudomallei*.** The identification of a centrally located gene with reverse transcriptional polarity relative to the other type II secretion genes was quite surprising (Fig. 2). We attempted to complement the secretion-deficient phenotype of DD214 in order to determine if it was due to the disruption of *gspC* or to a polar effect by other *gsp* genes located upstream and/or downstream of *gspC*. We amplified by PCR a 670-bp product encompassing *gspC* and cloned it into the broad-host-range plasmid pUCP28T. The pUCP28T derivative pIR2 was able to complement the mutation in DD214 in *trans* (Fig. 3B). The zones of clearing around 1026b(pUCP28T), 1026b(pIR2), and DD214(pIR2) on 3% skim milk agar were similar. As expected, the negative control DD214(pUCP28T) did not produce a zone of clearing on 3% skim milk agar. Similar results were obtained with tributyrin agar and egg yolk agar (data not shown). These results clearly demonstrate that the mutation in DD214 did not have a polar effect on upstream or downstream genes and that *gspC* is essential for the secretion of exoproducts in *B. pseudomallei*.

Relative virulence of 1026b and DD213. Syrian hamsters are highly susceptible to infection by *B. pseudomallei* (1, 4). The 50% lethal doses (LD₅₀) of 1026b and DD213 in the hamster model of infection were <5 and 13, respectively. These represent increases in LD₅₀ of 3- to 13-fold, depending on the actual LD₅₀ for 1026b. DD213 was reisolated from the blood and livers of several infected animals and was found to be Tc^r and deficient in exoproduct secretion, suggesting that the Tn5-

OT182 integration in this strain was stable in the absence of selection. These results suggest that exoproducts secreted by the type II pathway probably play a minor role in *B. pseudomallei* pathogenesis.

Nucleotide sequence accession number. The nucleotide sequences reported in this paper were deposited in the GenBank database under the accession no. AF110185 and AF110186.

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Benchmarks

Self-Cloning Minitransposon *phoA* Gene-Fusion System Promotes the Rapid Genetic Analysis of Secreted Proteins in Gram-Negative Bacteria

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Virulence determinants are inclined to be located on the bacterial surface or secreted into the milieu where they can interact with the host. A system that isolates defined mutations in secreted bacterial products is an invaluable tool for identifying virulence genes. One such system is the *phoA* gene-fusion approach that takes advantage of the fact that the normally periplasmic bacterial protein alkaline phosphatase (PhoA) is only biologically functional when secreted from the cytoplasm (2,4). Therefore, when a truncated form of *phoA*, lacking its signal sequence, inserts downstream of and in-frame with a signal sequence and forms a gene fusion, the exported fusion protein exhibits phosphatase activity.

Isolation of mutants carrying active PhoA-fusions is a simple procedure: colonies appear blue when grown on agar plates incorporated with the chromogenic substrate 5-bromo-4-chloro-3-indolyl phosphate (BCIP). The most widely used *phoA*-fusion system is the *TnphoA* transposon mobilized onto broad host range suicide plasmids such as pRT733 (4). Because of the broad host range of both delivery plasmids and *Tn5* transposon, this system is applicable to a wide range of Gram-negative bacterial species. However, *TnphoA* lacks convenient cloning sites, which results in the inefficient isolation of clones containing DNA flanking the transposon, and the isolated DNA is often composed of small segments. In addition, the presence of the cognate transposase within the inverted repeats can confer instability upon the transposon and induce further DNA rearrangements.

Here, we describe the construction of a Gram-negative, broad host range self-cloning minitransposon *phoA* gene-fusion system that markedly increases the efficiency of isolating clones containing DNA flanking the transposon. The sys-

tem also promotes the rapid isolation of large DNA fragments, allowing the isolation of clusters of virulence genes.

The broad host range plasmid-transposon construct pTnMod-OGm consists of a cognate transposase outside of the transposon's inverted repeats that permits the minitransposon to integrate into the target DNA without its transposase and prevents further DNA rearrangements (3). This system is constructed with a pUC (pMB1/ColIE1) conditional origin of replication within the transposon, which limits its replication to *Escherichia coli* and some closely related species (3). In addition, rare restriction endonuclease sites are incorporated near the inverted repeats, which

are features that promote the rapid cloning of DNA adjacent to the transposon's site of insertion. Oligonucleotide primers were designed based on the nucleotide sequence of the alkaline phosphatase gene without the signal sequence ('*phoA*') of *E. coli* and used to amplify a 1.7-kb product from pRT733 (*TnphoA*) (4). The sequence of the primers used are as follows (*KpnI* restriction sites are underlined): Pho-5', 5'-GCGGTACCCTGACTCTTATACA-CAAGTAGCGT-3' and Pho-3', 5'-GCGGTACCAGCAATCACTCATGTAGGT-3'. The 1.7-kb PCR product was digested with *KpnI* and ligated to the *KpnI* site of pTnMod-OGm creating the 6375-bp construct pmini-*OphoA*

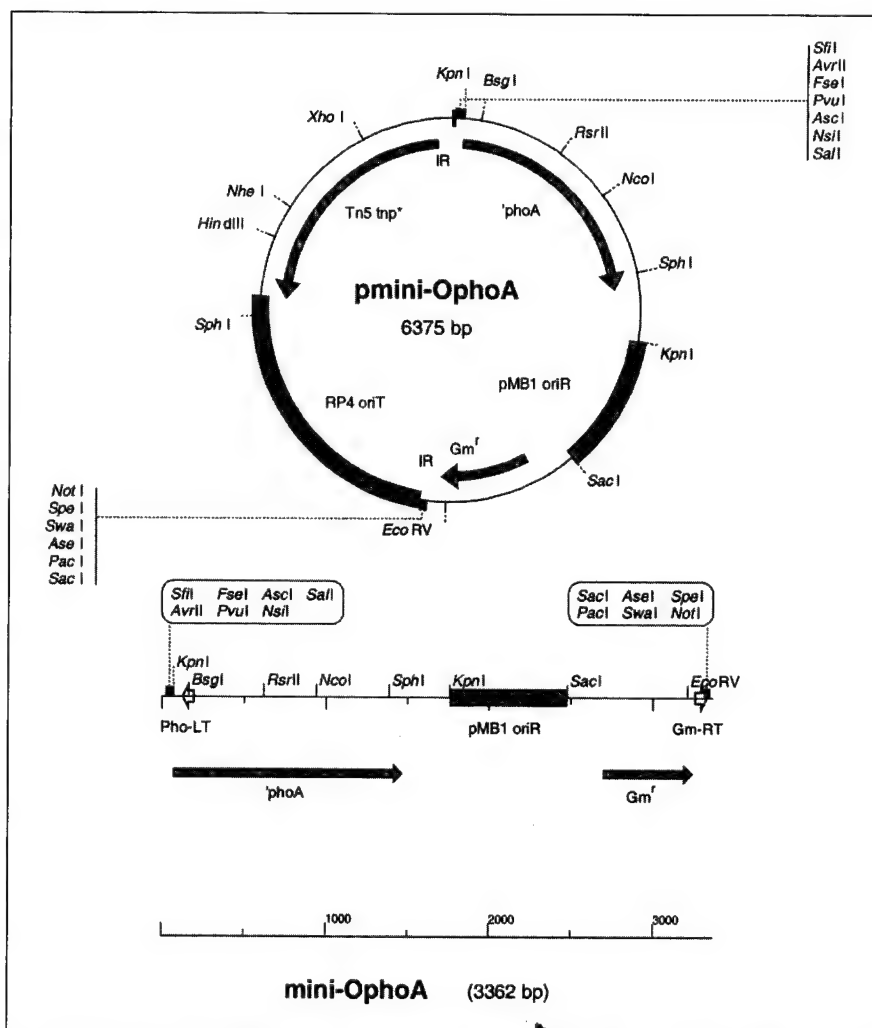


Figure 1. Schematic representation of the pmini-*OphoA* construct and the mini-transposon, mini-*OphoA*, following transposition into the host chromosome. '*phoA*', *E. coli* alkaline phosphatase without the signal sequence and expression signals; pMB1 oriR, narrow host range origin of replication; Gm^r, gentamicin resistance cassette; IR, *Tn5* inverted repeats; RP4 oriT, origin of transfer; *Tn5 tnp**, *Tn5* transposase; Pho-LT and Gm-RT, mini-*OphoA* universal sequencing primers (open arrows).

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(Figure 1). Orientation of the *'phoA* gene was confirmed following digestion of the construct with *SphI*. Insertion of *'phoA* does not disrupt its modular arrangement; the gentamicin resistance cassette can be released with *SacI* and replaced with alternative antibiotic cassettes, allowing the use of this system in bacteria that are naturally resistant to a variety of antibiotics (3).

The minitransposon derivative mini-*OphoA* (3362 bp) is smaller than *TnphoA* (7733 bp), and the absence of the cognate transposase confers a higher stability upon the transposon once it has integrated within the target DNA (Figure 1). The presence of the conditional origin of replication and multiple cloning sites within the inverted repeats promotes the efficient recovery of DNA flanking the transposon compared with other *TnphoA* systems (2,4). Sites for rare-cutting restriction enzymes within the multiple cloning sites will produce

clones that contain large segments of the genome. The close proximity of cloning sites greatly enhances sequencing of DNA flanking the transposon by virtue of the mini-*OphoA* universal primers, Pho-LT and Gm-RT.

The mini-*OphoA* universal primers are designed to nucleotide sequences adjacent to the multiple cloning sites (Figure 1). The sequence of the mini-*OphoA* universal primers are as follows: Pho-LT, 5'-CAGTAATATC-GCCCTGAGCAGC-3' and Gm-RT, 5'-GCCGCGGCCAATTCGAGCTC-3'. The *phoA*-fusion joint of flanking clones derived from *AseI*, *SpeI*, *PacI*, *SwaI* and *NotI* digests can be sequenced using the primer Pho-LT, and upstream DNA sequence can be determined using the Gm-RT primer. The converse can be applied when genomic DNA is digested with *SfiI*, *FseI*, *AscI*, *Sall*, *AvrII*, *PvuI* and *NsiI* when the primer Gm-RT is used to sequence the

transposon-fusion joint, and the downstream DNA sequence can be determined using the Pho-LT primer. The application of the mini-*OphoA* universal primers promotes the rapid sequencing of flanking DNA.

The plasmid-transposon construct was transferred into the nalidixic acid- and zeocin-resistant phosphatase-negative derivative of *Burkholderia mallei* strain ATCC 23344 by conjugation with *E. coli* SM10 (pmini-*OphoA*) and selected on agar incorporated with gentamicin (5 µg/mL), BCIP (80 µg/mL), zeocin (5 µg/mL) and nalidixic acid (75 µg/mL). Approximately 1000–2000 transconjugates were isolated per mutagenesis, with a frequency of phosphatase expressing (PhoA+) mutants of 2%. Chromosomal DNA was isolated from PhoA+ mutants by using the minichromosomal preparation protocol described by Ausubel et al. (1). One microgram of total genomic DNA was

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digested with either *NotI*, *SalI*, *AvrII*, *SpeI* or *PvuI*. Following digestion, the enzyme was heat inactivated, and the DNA was ethanol precipitated and resuspended in 20 μ L sterile distilled water. Ten microliters of this suspension were self-ligated in a total volume of 20 μ L overnight at 16°C. Four microliters of the resulting ligation mixture were subsequently transformed into 40 μ L competent *E. coli* DH5 α TM (Life Technologies, Rockville, MD, USA).

Isolation of both transconjugates and flanking clones was approximately ten-fold more efficient using pmini-*OphoA* than using the pRT733 (*TnphoA*) system. Flanking clones were sequenced using the mini-*OphoA* universal primers, Pho-LT and Gm-RT, which enabled sequencing of the *phoA*-fusion joint and DNA up or downstream of the transposon's site of insertion. Blastx searches of the PhoA-fusion proteins showed homology to secreted bacterial proteins and demonstrated that this system successfully isolates defined mutations in secreted proteins. The mini-*OphoA* system has been used successfully to isolate defined mutations in secreted proteins of *B. pseudomallei* and *B. thailandensis* (M.N. Burnick, A. Bolton, P. Brett, D. Watanabe and D.E. Woods, unpublished results).

Isolation of flanking clones that contain large segments of DNA can allow for the rapid identification of virulence loci because virulence-associated genes are often clustered. Moreover, sequencing of adjacent genes can often ascribe a putative function to the *phoA*-fusion protein and assign a foundation for virulence studies when *phoA*-fusion proteins exhibit no or low homology to other known proteins. An additional use of the pTnMod-OGm has been suggested by Dennis and Zylstra (3), who propose that plasmid-transposon construct-generated libraries are a relatively swift and simple alternative to the construction of cosmid libraries. This notion is based on the use of the rare-cutting restriction enzymes that will produce clones that contain large segments of the genome. This approach involves the isolation of genomic DNA from pools of TnMod-OGm transconjugates, cleavage with a rare-cutting restriction enzyme, self-ligation and transformation into a permissive *E. coli* host. A similar approach

could conceivably use mini-*OphoA* and confer the selection of libraries that encode secreted proteins.

In conclusion, we have constructed a broad host range self-cloning mini-transposon *phoA* gene-fusion system that promotes the rapid identification of defined mutations in secreted proteins of Gram-negative bacteria. The mini-*OphoA* is a versatile tool that enhances the isolation of virulence genes and promotes the recovery of large segments of DNA flanking the transposon, thus allowing the identification of clusters of virulence genes and providing an alternative approach for the production of cosmid libraries.

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Detection of Bacterial Virulence Genes by Subtractive Hybridization: Identification of Capsular Polysaccharide of *Burkholderia pseudomallei* as a Major Virulence Determinant

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Burkholderia pseudomallei, the etiologic agent of melioidosis, is responsible for a broad spectrum of illnesses in humans and animals particularly in Southeast Asia and northern Australia, where it is endemic. *Burkholderia thailandensis* is a nonpathogenic environmental organism closely related to *B. pseudomallei*. Subtractive hybridization was carried out between these two species to identify genes encoding virulence determinants in *B. pseudomallei*. Screening of the subtraction library revealed A-T-rich DNA sequences unique to *B. pseudomallei*, suggesting they may have been acquired by horizontal transfer. One of the subtraction clones, pDD1015, encoded a protein with homology to a glycosyltransferase from *Pseudomonas aeruginosa*. This gene was insertionally inactivated in wild-type *B. pseudomallei* to create SR1015. It was determined by enzyme-linked immunosorbent assay and immunoelectron microscopy that the inactivated gene was involved in the production of a major surface polysaccharide. The 50% lethal dose (LD₅₀) for wild-type *B. pseudomallei* is <10 CFU; the LD₅₀ for SR1015 was determined to be 3.5×10^5 CFU, similar to that of *B. thailandensis* (6.8×10^5 CFU). DNA sequencing of the region flanking the glycosyltransferase gene revealed open reading frames similar to capsular polysaccharide genes in *Haemophilus influenzae*, *Escherichia coli*, and *Neisseria meningitidis*. In addition, DNA from *Burkholderia mallei* and *Burkholderia stabilis* hybridized to a glycosyltransferase fragment probe, and a capsular structure was identified on the surface of *B. stabilis* via immunoelectron microscopy. Thus, the combination of PCR-based subtractive hybridization, insertional inactivation, and animal virulence studies has facilitated the identification of an important virulence determinant in *B. pseudomallei*.

Burkholderia pseudomallei, the causative agent of melioidosis, is a gram-negative, facultatively anaerobic, motile bacillus that is commonly found in the soil and stagnant waters in Southeast Asia and northern Australia. Infection by *B. pseudomallei* is often due to either direct inoculation into wounds and skin abrasions or to inhalation of contaminated material (11, 24, 30). This would explain the prevalence of the disease among rice farmers as well as helicopter pilots in the Vietnam War who developed melioidosis due to inhalation of contaminated dust (24, 47). Melioidosis may present as an acute pneumonia or an acute septicemia, which is the most severe form of the disease. The disease may also manifest as a chronic infection involving long-lasting suppurative abscesses in numerous sites in the body. Infection with *B. pseudomallei* may even result in a subclinical infection and remain undetected for a number of years. Both the chronic and subclinical forms generally remain undiagnosed until activated by a traumatic event or a decrease in immunocompetence (25).

Both secreted and cell-associated antigens have been identified in *B. pseudomallei*. Cell-associated antigens include exopolysaccharide (EPS) and lipopolysaccharide (LPS) (5, 8, 51). The EPS produced by *B. pseudomallei* is an unbranched polymer of repeating tetrasaccharide units with the structure -3)-

2-O-acetyl-β-D-Galp-(1-4)-α-D-Galp-(1-3)-β-D-Galp-(1-5)-β-D-KDop-(2- (35, 40). The role of EPS in virulence is unknown, but sera from patients with melioidosis have been shown to contain antibodies against EPS (51). The LPS of *B. pseudomallei* has been reported to contain two types of O-polysaccharide moieties termed type I O-PS and type II O-PS (27, 41). Type II O-PS is an unbranched heteropolymer with repeating D-glucose and L-talose residues with the structure -3)-β-D-glucopyranose-(1-3)-6-deoxy-α-L-talopyranose-(1-, in which approximately 33% of the talose residues contain 2-O-methyl and 4-O-acetyl substituents, while the other L-talose residues contain only 2-O-acetyl substituents. Type II O-PS has been shown to be involved in serum resistance (17). Mutants lacking in type II O-PS were found to be sensitive to the bactericidal activities of 30% normal human serum. Type II O-PS mutants also demonstrated reduced virulence in three animal models of *B. pseudomallei* infection (17). Type I O-PS is an unbranched homopolymer with the structure -3)-2-O-acetyl-6-deoxy-β-D-manno-heptopyranose-(1-. The role for this polysaccharide in infection was previously undefined.

B. thailandensis is a nonpathogenic soil organism originally isolated in Thailand (6). Based on biochemical, immunological, and genetic data, *B. pseudomallei* and *B. thailandensis* are closely related species. However, these two organisms differ in a number of ways and have been classified into two different species (7). The rRNA sequence of *B. thailandensis* differs from that of *B. pseudomallei* by 15 nucleotides, and there are significant differences in genomic macrorestriction patterns be-

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tween these organisms (10). The biochemical profiles of these two species differ in that *B. thailandensis* can utilize L-arabinose whereas *B. pseudomallei* does not (7, 62). The most distinct difference between these two species, however, is their relative virulence. The 50% lethal dose (LD_{50}) for *B. pseudomallei* in the Syrian hamster model of acute melioidosis is <10 organisms, whereas the LD_{50} for *B. thailandensis* is approximately 10^6 organisms (7). It has also been shown that the two species can be differentiated based on their propensity to cause disease in humans. Environmental strains isolated in Thailand that are able to assimilate L-arabinose are not associated with human infection, whereas clinical isolates are not able to utilize L-arabinose (54).

To identify the genetic determinants that confer enhanced virulence in *B. pseudomallei*, a method combining subtractive hybridization, insertional mutagenesis, and animal virulence studies was developed. The described method should aid in the identification of virulence factors in pathogenic bacteria and provide further insights into microbial diversity and evolution.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are described in Table 1. *B. pseudomallei*, *B. thailandensis*, *B. cepacia*, and *Escherichia coli* were grown at 37°C on Luria-Bertani (LB) broth base (Becton Dickinson) agar plates or in LB broth. *B. mallei* was grown at 37°C on LB plates or in LB broth supplemented with 4% glycerol and at pH 6.8. For animal studies, *B. pseudomallei* and *B. thailandensis* cultures were grown at 37°C in TSBDC medium (6). When appropriate, antibiotics were added at the following concentrations: 50 µg of tetracycline, 100 µg of streptomycin, 100 µg of polymyxin B, 100 µg of trimethoprim, 25 µg of gentamicin, and 25 µg of kanamycin per ml for *B. pseudomallei* and 100 µg of ampicillin, 25 and 50 µg of kanamycin, 15 µg of tetracycline, and 1.5 mg of trimethoprim per ml for *E. coli*.

Construction and screening of subtractive hybridization libraries. Subtractive hybridization between *B. pseudomallei* and *B. thailandensis* was carried out using a PCR-Select bacterial genome subtraction kit (Clontech) as recommended by the manufacturer except that the hybridization temperature was increased from 63°C to 73°C due to the high G+C content in the genomes of these species. In construction of the subtractive hybridization library, *B. pseudomallei* genomic DNA was used as the tester and *B. thailandensis* genomic DNA was used as the driver. The secondary PCR products obtained were cloned into pZerO-2.1 (Invitrogen) and pPCR (Table 1) and were enriched for *B. pseudomallei*-specific sequences. The subtraction library was screened by sequencing of the tester-specific DNA fragments. The library containing random clones was diluted in sterile phosphate-buffered saline (PBS) to 10^{-6} , and 100 µl was plated on LB plates containing kanamycin (50 µg/ml) and 1 mM isopropylthio-β-D-galactoside (IPTG). Individual colonies were picked and grown overnight at 37°C in LB with kanamycin (50 µg/ml). Plasmid DNA was isolated using a miniprep plasmid isolation kit (Qiagen).

DNA sequencing and analysis. Automated DNA sequencing was performed by ACGT (Northbrook, Ill.) and the University of Calgary Core DNA Services. The M13 forward primer (dGTAAACGACGGCCAGT) was used to initiate sequence reactions with the subtractive hybridization clones. DNA flanking the Tn5-OT182 insertions was sequenced using the previously described primers OT182-LT and OT182-RT (16). The DNA flanking the insertion of pSR1015 was sequenced using the pSKM11 primer (38). DNA and protein sequences were analyzed with DNASIS for IBM and with the ORF Finder program at the National Center for Biotechnology Information (NCBI). DNA sequences were analyzed for homology using the BLASTX program through GenBank at NCBI.

Cloning of a subtractive hybridization product and mobilization into wild-type *B. pseudomallei*. The DNA insert from pDD1015 was cloned as a *KpnI-XhoI* fragment into a mobilizable suicide vector, pSKM11 (Table 2). The 373-bp fragment was ligated to pSKM11 digested with the same enzymes to create pSR1015. SM10(pSR1015) was conjugated with *B. pseudomallei* 1026b using a previously described protocol (16).

Animal studies. The animal model of acute *B. pseudomallei* infection has been previously described (18). Syrian hamsters (females, 6 to 8 weeks) were injected intraperitoneally with 100 µl of one of a number of serial dilutions of logarithmic-phase cultures in sterile PBS. The five control animals were inoculated with

10^1 CFU of wild-type *B. pseudomallei*. The test animals (five per dilution) were inoculated with either 10^1 , 10^2 , or 10^3 CFU of the mutant strain, SR1015. Blood from two of the test animals was diluted and plated on Ashdown medium with and without the addition of tetracycline (50 µg/ml) to verify the stability of pSR1015 (7). For determination of LD_{50} for SR1015, hamsters (five per group) were inoculated with 10^3 , 10^4 , 10^5 , and 10^6 CFU. After 48 h, the LD_{50} was calculated (42).

Immunassays. Immunogold electron microscopy was performed as previously described (17). Samples for Western blot analysis were prepared as previously described (8). Immunoassay was performed with a 1:250 dilution of the primary antibody, polyclonal rabbit antiserum raised to a *B. pseudomallei* O-PS-flagellin protein conjugate (5, 8). The secondary antibody used was horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG) (Sigma). Enzyme-linked immunosorbent assays (ELISAs) for the presence of EPS were carried out as previously described, using the EPS-specific monoclonal antibody 3015 at a dilution of 1/100 (17, 51, 52). Tn5-OT182 mutants were screened by ELISA according to the same protocol with rabbit polyclonal sera specific for an O-PS-flagellin conjugate containing antibodies to type I O-PS, type II O-PS, and flagella (5).

Southern hybridization. For Southern hybridization, *SstI* digests of genomic DNA from *B. pseudomallei* 1026b and SR1015, *B. thailandensis* E264, *B. mallei* NCTC 10260, *B. cepacia* CEP509 (genomovar I) and K56-2 (genomovar III), *B. siabilis* LMG14294 and LMG7000, *B. vietnamiensis* LMG10929, and *B. multivorans* C5393 were transferred to GeneScreen Plus membranes (Du Pont Canada, Lachine, Quebec, Canada), and hybridization was performed at 65°C in 15 ml of 1% sodium dodecyl sulfate (SDS)-10% dextran sulfate-salmon sperm DNA (0.1 mg/ml) according to the manufacturer's recommendations. The 0.4-kb *KpnI-XhoI* fragment from pDD1015 was used as a probe and labeled with [32 P]dCTP using an oligonucleotide labeling kit (Pharmacia Biotech, Inc., Baie d'Urfe, Quebec, Canada).

Tn5-OT182 mutagenesis and screening for type I O-PS mutants. To screen for mutants deficient in type I O-PS, it was first necessary to create a strain that produced only type I O-PS. This was necessary as the antiserum available was polyclonal antiserum to a flagellin-O-PS conjugate that contains antibodies to flagellin, type I O-PS, and type II O-PS. Therefore, we constructed a strain that was lacking in type II O-PS and flagella, SR1001. Allelic exchange was carried out using two strains that were previously constructed in the laboratory. The donor strain, SM10 λ pir(pPB611::Gm), has a plasmid containing a copy of the *whiE* gene, involved in the synthesis of type II O-PS, which has been mutated by the insertion of a gentamicin resistance (Gm) cassette (17). The recipient strain, PB401, is a *B. pseudomallei* strain that has a deletion in the *flhC* gene. SM10 λ pir(pPB611::Gm) was conjugated to PB401, and transconjugants were selected for by plating on LB containing gentamicin, kanamycin, and polymyxin B. Transconjugants that were Sm r , Gm r , and Km r were selected (to select for loss of the vector, pKAS46), and one was designated SR1001. Transposon mutagenesis of SR1001 was performed with Tn5-OT182 according to a previously described protocol (16) except that transconjugants were selected on plates containing gentamicin and tetracycline. Transposon mutants were inoculated into 96-well plates containing 200 µl of LB with gentamicin and tetracycline and grown overnight at 37°C at 250 rpm. A negative growth control well was included for each plate. The wells of a 96-well plate were coated with 10 µl of bacteria and 90 µl of coating buffer (0.05 M carbonate buffer [pH 9.6]), and ELISA was carried out as previously described (5). The primary antibody, polyclonal rabbit antiserum to a *B. pseudomallei* O-PS-flagellin protein conjugate, was added at a dilution of 1:1,000. The secondary antibody, a goat anti-rabbit IgG-peroxidase conjugate (Sigma), was added at a dilution of 1:1,000. The plates were developed with an HRP color development reagent (Bio-Rad Laboratories) for 30 min. The optical density at 405 nm (OD_{405}) was determined using an ELISA reader. *B. pseudomallei* 1026b was included as a positive control, and *E. coli* DH5α was included as a negative control. Transposon mutants that had OD_{405} readings comparable to the negative control ($OD_{405} = <0.100$) were chosen for further analysis.

Construction of allelic exchange mutants. Allelic exchange was carried out as previously described (17). The allelic exchange vector used in these experiments was pKAS46, an allelic exchange vector based on *rpsL* for counterselection (49). *B. pseudomallei* DD503, a double mutant that contains the $\Delta amrR-oprA$ and *rpsL* mutations, was the recipient strain used for all allelic exchange experiments (17, 39). All genes in these experiments were mutated by the insertion of a self-cloning trimethoprim resistance (Tp) cassette from p34EoriTp (P. J. Brett, D. DeShazer, and D. E. Woods, unpublished data). For each allelic exchange experiment, SM10 λ pir transformed with pKAS46 containing the mutated allele was conjugated to *B. pseudomallei* DD503 as described above for the construction of SR1001 except that the transconjugants were plated on polymyxin B,

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Strains		
<i>E. coli</i>		
SM10	Mobilizing strain; transfer genes of RP4 integrated in chromosome; Km ^r Sm ^s	48
SM10 λ pir	SM10 with a λ prophage carrying the gene encoding the π protein	37
SURE	e14 ⁻ (<i>mcrA</i>) Δ (<i>mcrCB-hsdSMR-mrr</i>)171 <i>endA1 supE44 thi-1 gyrA96 relA1 lac recB recJ sbcC umuC::Tn5 uvrC</i> [F' <i>proAB lacI^s Z</i> Δ M15 Tn10] Kan ^r Tet ^r	Stratagene
DH5 α	F ⁻ ϕ 80 <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>endA1 recA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1</i>	Bethesda Research Laboratories
TOP10	F ⁻ <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80 <i>lacZ</i> Δ M15 Δ <i>lacX74 deoR recA1 araD139</i> Δ (<i>ara-leu</i>)7697 <i>galU galK rpsL</i> (Str ^r) <i>endA1 nupG</i>	Invitrogen
XL10-Gold	Tet ^r Δ (<i>mcrA</i>)183 Δ (<i>mcrCB-hsdSMR-mrr</i>)173 <i>endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte</i> [F' <i>proAB lacI^s Z</i> Δ M15 Tn10 (Tet ^r) Amy Cam ^r]	Stratagene
<i>B. pseudomallei</i>		
1026b	Clinical isolate; Km ^r Gm ^r Sm ^r Pm ^r Tc ^s Tp ^s	16
SR1015	1026b(pSR1015); Sm ^r Tc ^r	This study
SR1016	1026b(pSR1016); Sm ^r Tc ^r	This study
DD503	1026b derivative; allelic exchange strain; Δ (<i>amrR-oprA</i>)(Km ^s Gm ^s Sm ^s) <i>rpsL</i> (Sm ^r)	39
PB401	DD503 derivative; Δ <i>fliC</i>	Brett et al., unpublished
SR1001	DD503 derivative; Δ <i>fliC wbiE::aacC1</i> Gm ^r	This study
SLR5	SR1001 derivative; <i>wcbB::Tn5-OT182</i> Tc ^r	This study
SLR8	SR1001 derivative; <i>wzt2::Tn5-OT182</i> Tc ^r	This study
SLR13	SR1001 derivative; <i>wcbP::Tn5-OT182</i> Tc ^r	This study
SLR18	SR1001 derivative; <i>wcbE::Tn5-OT182</i> Tc ^r	This study
SLR19	SR1001 derivative; <i>wcbH::Tn5-OT182</i> Tc ^r	This study
SR201::Tp	DD503 derivative; <i>wcbC::Tp</i>	This study
SR202::Tp	DD503 derivative; <i>wcbA::Tp</i>	This study
SR203::Tp	DD503 derivative; <i>yafJ::Tp</i>	This study
<i>B. thailandensis</i> E264	Soil isolate; LPS contains only type II O-PS	7
<i>B. mallei</i> NCTC 10260		
<i>B. cepacia</i> complex		
<i>B. cepacia</i> CEP509 (genomovar I)	CF ^a isolate, Australia	33
<i>B. multivorans</i> C5393 (formerly <i>B. cepacia</i> genomovar II)	CF isolate, Vancouver, Canada	33
<i>B. cepacia</i> K56-2 (genomovar III)	CF isolate, Toronto, Canada	15
<i>B. stabilis</i> (formerly <i>B. cepacia</i> genomovar IV)		
CEP0717	CF isolate, Calgary, Canada	H. Rabin
CEP0467	CF isolate, Edmonton, Canada	E. Mahenthiralingam
J687	Non-CF isolate, France	56
CEP0726	CF isolate, Calgary, Canada	H. Rabin
LMG14291	CF isolate, Belgium	56
LMG7000	Blood isolate, Sweden	56
LMG14294	CF isolate, Belgium	33
<i>B. vietnamiensis</i> (also known as <i>B. cepacia</i> genomovar V)	Rice root isolate, Vietnam	33
LMG10929		
Plasmids		
pSKM11	Positive selection cloning vector; IncP <i>mob</i> ; ColE1 <i>ori</i> ; Ap ^r Tc ^s	38
pPCR	pBluescript II SK(+) derivative; Ap ^r	3
pZER0-2.1	Positive selection cloning vector; ColE1; Km ^r	Invitrogen
pPCR2.1-TOPO	Topoisomerase-mediated cloning vector; Ap ^r Km ^r	Invitrogen
pDD1015	Subtractive hybridization product cloned into pZER0-2.1; Km ^r	This study
pDD1016	Subtractive hybridization product cloned into pZER0-2.1; Km ^r	This study
pSR1015	<i>KpnI-XhoI</i> fragment from pDD1015 cloned into pSKM11 Ap ^r Tc ^r	This study
pSR1016	<i>KpnI-XhoI</i> fragment from pDD1016 cloned into pSKM11; Ap ^r Tc ^r	This study
pSR1015Bg	8-kb <i>BglII</i> fragment from SR1015 obtained by self-cloning; Ap ^r Tc ^r	This study
pOT182	pSUP102(Gm)::Tn5-OT182; Cm ^r Gm ^r Ap ^r Tc ^r	36
pSLR5B	8-kb <i>BamHI</i> fragment from SLR5 obtained by self-cloning; Ap ^r	This study
pSLR5H	10-kb <i>HindIII</i> fragment from SLR5 obtained by self-cloning; Ap ^r	This study
pSLR13H	9-kb <i>HindIII</i> fragment from SLR13 obtained by self-cloning; Ap ^r	This study
p34E-oriTp	Vector containing self-cloning Tp cassette; <i>dhfrIIIb-p15A oriV</i>	Brett et al., unpublished

^a CF, cystic fibrosis.

TABLE 2. Recombinant plasmids in the *B. pseudomallei*-*B. thailandensis* subtraction library

Plasmid	Vector	Insert size (bp)	% G+C	Homologue ^a
pDD1000	pPCR	326	51	DprA
pDD1001	pPCR	800	44	None
pDD1002	pPCR	434	50	GuaA
pDD1003	pPCR	346	51	None
pDD1004	pPCR	800	44	None
pDD1005	pPCR	531	46	Mob protein
pDD1006	pPCR	353	48	None
pDD1007	pZErO-2.1	325	51	None
pDD1008	pZErO-2.1	250	44	None
pDD1009	pZErO-2.1	350	52	None
pDD1012	pZErO-2.1	505	47	None
pDD1015	pZErO-2.1	373	52	WbpX
pDD1016	pZErO-2.1	259	46	None
pDD1017	pZErO-2.1	100	50	None
pDD1018	pZErO-2.1	433	50	None

^a Homologues and references: *Haemophilus influenzae* DprA, Karudapuram et al. (26); *Bacillus subtilis* GuaA, Mantsala and Zalkin (34); *Bordetella bronchiseptica* plasmid pBBR1 mobilization (Mob) protein, Antoine and Loch (1); *Pseudomonas aeruginosa* WbpX, Rocchetta et al. (45).

kanamycin, and trimethoprim. The Pm^r Km^r Tp^r transconjugants were subsequently transferred to plates containing streptomycin to select for the loss of pKAS46. Mutant alleles were confirmed by self-cloning and sequencing.

DNA manipulation. Restriction enzymes and T4 DNA ligase were purchased from Life Technologies (Burlington, Ontario, Canada) and New England Biolabs (Mississauga, Ontario, Canada) and used according to the manufacturer's instructions. DNA fragments used in cloning procedures were excised from agarose gels and purified using a GeneClean II kit (Bio 101, Vista, Calif.) or Qiagen (Mississauga, Ontario, Canada) gel extraction kit. Chromosomal DNA was isolated using a previously described protocol (60). The self-cloning of *B. pseudomallei* flanking DNA from Tn5-OT182 mutants and from SR1015 was performed as described previously (16).

Nucleotide sequence accession number. The nucleotide sequence reported in this paper has been deposited in the GenBank database under accession number AF228583.

RESULTS

Construction and screening of the *B. pseudomallei* subtraction library. Subtractive hybridization was carried out between the virulent *B. pseudomallei* and the weakly virulent *B. thailandensis* in order to isolate DNA sequences encoding for virulence determinants unique to *B. pseudomallei*. The genomic DNA sample from *B. pseudomallei* containing the sequences of interest was known as the tester DNA, and genomic DNA from *B. thailandensis*, the reference sample, was called the driver DNA. Tester and driver DNAs were digested and subjected to two rounds of hybridization. The remaining unhybridized sequences were considered tester-specific sequences. To enrich for tester-specific sequences, excess driver DNA was added in the hybridizations. The tester-specific sequences were then amplified by PCR and cloned into pPCR or pZErO-2.1 (Table 1).

Screening of the subtraction library revealed a number of DNA sequences unique to *B. pseudomallei*. Fifteen distinct plasmid inserts from the library were sequenced (Table 2). The DNA inserts ranged from 100 to 800 bp in length and were found to contain an average G+C content of approximately 44 to 52%, which is considerably lower than the 68% G+C content of the *B. pseudomallei* chromosome. The DNA sequences were analyzed using the NCBI BLASTX program, and only four of the sequences had homology to predicted proteins

present in the GenBank database. One of the plasmid inserts, pDD1000, had homology to DprA, a protein required for chromosomal DNA transformation in *Haemophilus influenzae* (26). Another insert, pDD1005, had homology to a mobilization protein found in small plasmids (1). The third, pDD1015, was found to share limited homology with WbpX, a glycosyltransferase, from *Pseudomonas aeruginosa* (45). The fourth, pDD1002, demonstrated homology to GuaA, a GMP synthetase, from *Bacillus subtilis* (34).

Insertional inactivation of the glycosyltransferase gene in wild-type *B. pseudomallei*. The 373-bp DNA insert from pDD1015 was cloned into a mobilizable suicide vector, pSKM11 (Table 1). The resulting plasmid, pSR1015, was mobilized into wild-type *B. pseudomallei* 1026b to create the mutant strain SR1015. Since the insert from pDD1015 was found to demonstrate homology to a glycosyltransferase from *P. aeruginosa*, it was postulated that it might encode a protein involved in carbohydrate synthesis.

To define the phenotype of SR1015, an ELISA was performed with the EPS-specific monoclonal antibody 3015, and *B. pseudomallei* 1026b and SR1015 were both found to contain EPS (data not shown) (52). SR1015 was also shown to contain type II O-PS and to be serum resistant (data not shown). Immunogold electron microscopy studies using rabbit polyclonal sera specific for a type I O-PS-flagellin conjugate was performed on the parent strain, 1026b, and SR1015 (Fig. 1). *B. pseudomallei* 1026b reacted with antibodies to both flagellin and type I O-PS, as was evident by the distribution of gold particles around the bacterial surface and extending out along the flagella (Fig. 1A). The distribution of the gold particles around the outer surface of the bacteria corresponds to the type I O-PS structure, which is known to extend out beyond the type II O-PS. Unlike *B. pseudomallei* 1026b, SR1015 reacted only with the antibodies to flagellin, as the gold particles were found associated only with the flagella (Fig. 1B). *B. thailandensis*, the negative control, did not react with the antibodies either to flagellin or to type I O-PS (Fig. 1C). Western blot analysis of proteinase K-digested whole cells from *B. pseudomallei* 1026b, *B. thailandensis* E264, and *B. pseudomallei* SR1015 using rabbit polyclonal sera raised to O-PS-flagellin protein conjugate confirmed the lack of type I O-PS in SR1015 (Fig. 2). Type I and type II O-PS were stained in *B. pseudomallei* 1026b, while only type II O-PS was stained in the lanes corresponding to *B. pseudomallei* SR1015 and *B. thailandensis*. These results indicated that we had identified and insertionally inactivated a gene involved in the synthesis of the type I O-PS of *B. pseudomallei*.

SR1015 is avirulent in the animal model of infection. SR1015 was tested for virulence in the Syrian hamster model of acute septicemic melioidosis. The LD₅₀ for SR1015 after 48 h was 3.5×10^5 CFU, while the LD₅₀ of the parent strain, 1026b, was <10 CFU. The LD₅₀ for SR1015 was similar to that for the weakly virulent *B. thailandensis* (6.8×10^5 CFU). This demonstrates that SR1015 is severely attenuated for virulence in this animal model of melioidosis and that type I O-PS is a major virulence determinant of *B. pseudomallei*.

Cloning and sequencing of the genetic loci required for type I O-PS production and export. Two methods were used to clone the genes involved in the production and export of type I O-PS. The DNA flanking the insertion of pSR1015 was cloned from SR1015 and sequenced. We also used transposon

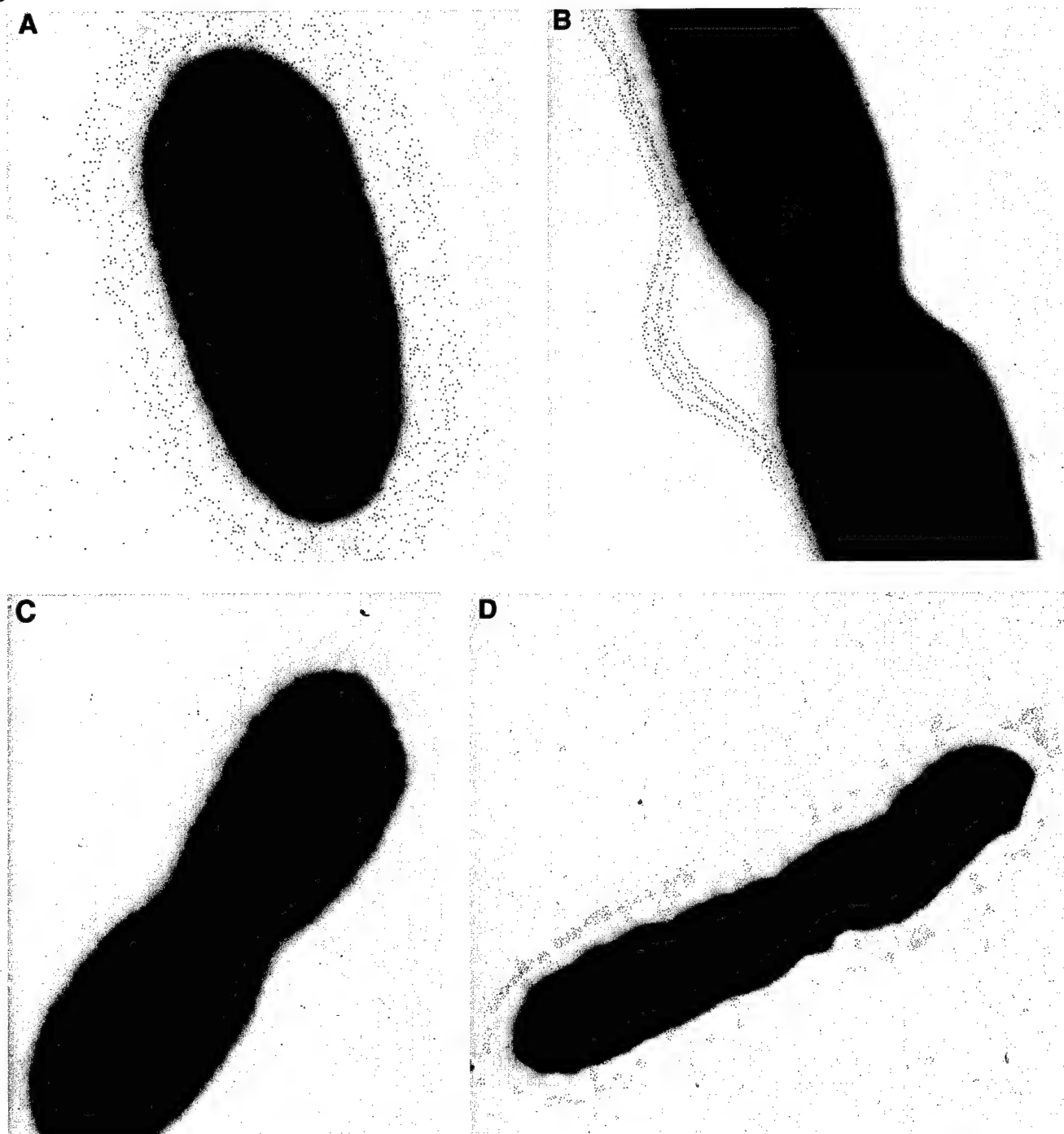


FIG. 1. Immunogold electron microscopy of *B. pseudomallei* 1026b (A) and SR1015 (B), *B. thailandensis* E264 (C), and *B. stabilis* LMG7000 (D). Bacteria were reacted with polyclonal rabbit antiserum directed against an O-PS-flagellin protein conjugate absorbed with *B. thailandensis* E264 to remove the antibodies directed against type II O-PS, washed, and reacted with a goat anti-rabbit IgG-gold (5 nm) conjugate. Original magnification, $\times 30,000$.

mutagenesis to clone the genes involved in production of the polysaccharide; this was done to obtain any unlinked genes that may be involved in polysaccharide production. Approximately 1,300 transposon mutants were screened for loss of type I O-PS by ELISA. Six mutants were identified, and the DNA flanking the transposon insertion was cloned and sequenced.

The Tn5-OT182 mutants SLR5, SLR8, SLR13, SLR18, and SLR19 mapped to the same region of the chromosome (Fig. 3). Sequence analysis of the cloned fragments revealed the presence of 20 potential open reading frames involved in the synthesis and export of type I O-PS (Fig. 3). The open reading frames that predicted proteins involved in polysaccharide bio-

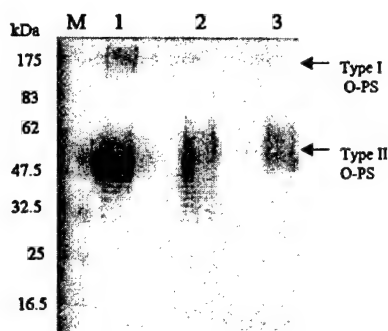


FIG. 2. Western blot analysis of LPS isolated from *B. pseudomallei* 1026b and SR1015 and *B. thailandensis* E264. Bacteria were reacted with proteinase K, subjected to SDS-polyacrylamide gel electrophoresis, electroblotted, and reacted with polyclonal rabbit antiserum raised to an O-PS-flagellin protein conjugate from *B. pseudomallei*. Lane M, prestained protein molecular weight standards (New England Biolabs); lane 1, *B. pseudomallei* 1026b; lane 2, *B. pseudomallei* SR1015; lane 3, *B. thailandensis* E264. The apparent molecular masses of the prestained proteins are indicated.

synthesis were found to demonstrate homology to proteins involved in the synthesis of a polysaccharide structure composed primarily of mannose (Table 3). The other reading frames in the locus predicted proteins involved in the transport of capsular polysaccharides in a variety of bacteria, particularly those that produce group 2 and group 3 capsular polysaccharides (Table 3 and reference 59). The genes responsible for the production of type I O-PS was found to be similar to other loci encoding for capsular polysaccharides in that they are divergently transcribed (Fig. 3 and reference 44). The gene cluster involved in the production of this polysaccharide is also similar to group 3 capsule gene clusters in that there are no genes encoding KpsF and KpsU, which are present in group 2 capsule gene clusters (59). However, the organization of the *B. pseudomallei* type I O-PS gene cluster differs in that it does not contain two export regions flanking a single biosynthetic region as seen in other group 3 capsule polysaccharide clusters (12). The biosynthetic genes identified thus far are not organized into one continuous transcriptional unit; instead, *wcbB*, *manC*, and *wcbP* are separated from the rest of the biosynthetic genes.

Another interesting feature is that *kpsC* is usually found next to *kpsS* in other group 2 and 3 clusters, unlike the case for *wcbA* and *wcbO* in *B. pseudomallei* (Fig. 3 and reference 59). The promoter sequences of the transcriptional regions of the type I O-PS cluster have yet to be identified. The overall G+C content of this region is about 58%, lower than the G+C content of the rest of the chromosome (68%). The low G+C content in these clusters suggests that polysaccharide genes have a common origin and may have been transferred horizontally between species (21).

The genes involved in the production of the type I O-PS have been named according to the bacterial polysaccharide gene nomenclature scheme (43). The gene products associated with the type I O-PS cluster and their homologues are listed in Table 3. Mutations constructed in a number of these genes have confirmed their role in the production of type I O-PS. One gene that is required for the production of the polysaccharide is *wcbA* (Fig. 3; Table 3). The *wcbA* gene and *wcbO* predict proteins that demonstrate homology to the KpsC and KpsS proteins of *E. coli* and the LipA and LipB proteins of *Neisseria meningitidis*, respectively (Table 3). These proteins are involved in the processing and export of capsular polysaccharide in these organisms (22, 44). To confirm the role of the *wcbA* gene in capsule production, an allelic exchange mutant was constructed by the insertion of a Tp cassette (Fig. 3). The resulting strain, SR202::Tp, did not produce polysaccharide and demonstrated attenuated virulence in the hamster model, similar to SR1015 (data not shown).

The *wcbC*, *wcbD*, *wzm2*, and *wzt2* genes encode proteins that demonstrate homology to proteins involved in the transport of capsular polysaccharides (Table 3) (20, 28, 46). The *wcbC* gene predicts a protein that shares homology with KpsD, a periplasmic protein involved in capsule polysaccharide export in *E. coli* (21, 28, 61). An isogenic mutant was constructed by the insertion of a Tp cassette into the *wcbC* gene (Fig. 3). The resulting strain, SR201::Tp, was still virulent in the hamster model, and type I O-PS was detected on Western blots (data not shown). This is in contrast to the phenotype observed with *E. coli* *kpsD* mutants (61). The gene products encoded by *wzm2* and *wzt2* are homologous to the KpsM and KpsT proteins of *E. coli*, CtrC and CtrD of *N. meningitidis*, and BexA and BexB of *H.*

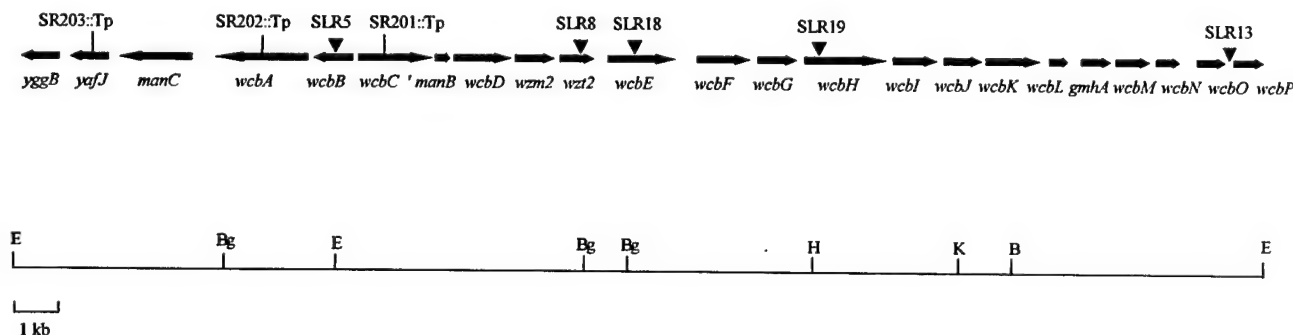


FIG. 3. Organization of the chromosomal region containing the genes responsible for the synthesis and export of type I O-PS in *B. pseudomallei*. The upper part shows the locations of the genes. The direction of transcription is represented by arrows, and gene names are indicated. The locations of Tn5-OT182 insertions are represented by triangles. Mutants constructed by allelic exchange are shown. The straight line indicates insertion of the Tp cassette into the gene of interest. The horizontal line below the genetic map represents *B. pseudomallei* chromosomal DNA; the locations of relevant restriction endonuclease recognition sites (Bg, *BglII*; E, *EcoRI*; H, *HindIII*; K, *KpnI*; B, *BamHI*) are shown.

TABLE 3. Genes involved in the production and export of type I O-PS in *B. pseudomallei* and homologous proteins located in the nonredundant sequence database

Gene	Size (bp)	Homologue, strain	Putative function	% Identity	% Similarity
<i>manC</i>	1,427	ManC, <i>Escherichia coli</i>	Mannose-1-phosphate guanylttransferase	55	70
		ManC, <i>Salmonella enterica</i> serovar Typhimurium		54	68
		ManC, <i>Klebsiella pneumoniae</i>		48	65
<i>wcbA</i>	2,015	KpsC, <i>E. coli</i>	Capsule polysaccharide export protein	37	52
		LipA, <i>Neisseria meningitidis</i>		39	55
		PhyA, <i>Pseudomonas multocida</i>		35	51
<i>wcbB</i>	1,097	WbpX, <i>P. aeruginosa</i>	Glycosyltransferase/mannosyltransferase	33	49
		ManB, <i>Aquifex aeolicus</i>		26	44
		MtfA, <i>Archaeoglobus fulgidis</i>		30	47
<i>wcbC</i>	1,163	KpsD, <i>E. coli</i>	Capsule export periplasmic protein	26	39
		CtrA, <i>N. meningitidis</i>		40	58
		BexD, <i>Haemophilus influenzae</i>		37	58
<i>'manB</i>	203	XanA, <i>Xanthomonas campestris</i>	Phosphomannomutase	35	48
		ManB, <i>K. pneumoniae</i>		29	49
		Rfk9, <i>E. coli</i>		35	53
<i>wcbD</i>	1,148	BexC, <i>H. influenzae</i>	Capsule export inner membrane protein	40	62
		CtrB, <i>N. meningitidis</i>		38	60
		KpsE, <i>E. coli</i>		26	48
<i>wzm2</i>	410	CtrC, <i>N. meningitidis</i>	Capsule export inner membrane protein	53	73
		BexB, <i>H. influenzae</i>		28	50
		KpsM, <i>E. coli</i>		28	50
<i>wzt2</i>	746	BexA, <i>H. influenzae</i>	ATP-binding protein	59	75
		CtrD, <i>N. meningitidis</i>		57	72
		KpsT, <i>E. coli</i>		46	66
<i>wcbE</i>	1,523	MtfB, <i>A. aeolicus</i>	Mannosyltransferase/glycosyltransferase	28	42
		WbpX, <i>P. aeruginosa</i>		38	55
		ManB, <i>Synechocystis</i> spp.		30	48
<i>wcbF</i>	1,379	Putative, <i>Homo sapiens</i>	Heparan-sulfate 6-sulfotransferase	24	39
		Putative <i>Arabidopsis thaliana</i>	En/Spm transposon protein	37	52
<i>wcbG</i>	941	SyfB, <i>Helicobacter pylori</i>	Phenylalanyl-tRNA synthetase	29	46
<i>wcbH</i>	1,796	MtfA, <i>Archaeoglobus</i> spp.	Mannosyltransferase/glycosyltransferase	23	40
		Putative, <i>Synechocystis</i> spp.		25	42
		Putative, <i>Streptomyces coelicolor</i>		28	41
<i>wcbI</i>	1,187	NifQ, <i>Enterobacter agglomerans</i>	Nitrogen fixation protein	28	41
<i>wcbJ</i>	842	Rbd1, <i>Methanobacterium thermoautotrophicum</i>	dTDP-4-dehydrorhamnose reductase	23	39
		RmlD, <i>Mycobacterium tuberculosis</i>		24	40
<i>wcbK</i>	1,013	Gm4D, <i>E. coli</i>	GDP-mannose dehydratase	29	48
		Gm4D, <i>Yersinia pseudotuberculosis</i>		32	49
		Gm4D, <i>Vibrio cholerae</i>		30	48
<i>wcbL</i>	1,040	Putative, <i>Campylobacter jejuni</i>	Sugar kinase	40	56
		Rv0115, <i>M. tuberculosis</i>	Lincomycin production	39	52
		LmbP, <i>Synechocystis</i> spp.		24	36
<i>gmhA</i>	593	LpcA, <i>H. pylori</i>	Phosphoheptose isomerase	50	68
		GmhA, <i>Methanococcus jannaschii</i>		54	72
		LpcA, <i>H. influenzae</i>		45	60
<i>wcbM</i>	692	RmlA2, <i>M. tuberculosis</i>	Mannose-1-phosphate guanylttransferase	32	46
		Putative, <i>C. jejuni</i>	Sugar-phosphate nucleotidyltransferase	41	58
<i>wcbN</i>	353	YaeD, <i>E. coli</i>	Hypothetical intergenic protein	40	62
		YaeD, <i>H. influenzae</i>	Hypothetical intergenic protein	40	58
<i>wcbO</i>	731	KpsS, <i>E. coli</i>	Capsule polysaccharide export protein	34	46
		PhyB, <i>Pasteurella multocida</i>		29	45
		LipB, <i>N. meningitidis</i>		27	44
<i>wcbP</i>	1,950	YooP, <i>M. tuberculosis</i>	Oxidoreductase	37	52
		HetN, <i>Anabaena</i> spp.		34	52

influenzae (Table 3). These proteins are ATP-binding cassette (ABC) transporters that comprise an inner membrane polysaccharide export system (50). The *wzm2* and *wzt2* gene products of *B. pseudomallei* likely comprise an ABC transporter system that is involved in the transport of the type I O-polysaccharide across the cytoplasmic membrane. The termination codon of the *wzm2* gene overlaps the initiation codon of the *wzt2* gene, suggesting that these two genes are translationally coupled. The *kpsM* and *kpsT* genes of *E. coli* are organized into a single transcriptional unit, and both genes are translationally coupled (44). These genes have been designated *wzm2* and *wzt2* since *wzm* and *wzt* have previously been identified and are associated with the type II O-PS gene cluster (17). A hydrophobicity plot of the predicted *wzm2* gene product revealed a hydrophobic protein with multiple-membrane spanning domains, like KpsM, that may act as an integral membrane protein for the export of polysaccharide (29). Analysis of the primary amino acid sequence of the predicted Wzt2 protein from *B. pseudomallei* has shown that this protein contains a conserved ATP-binding motif, including an A site (GGNGAG KST) and a B site (DCFLIDE) (57). The *wzt2* gene was found to be necessary for the production of type I O-PS in *B. pseudomallei*. In SLR18, the insertion of Tn5-OT182 in the *wzt2* gene resulted in a loss of type I O-PS.

The *wcbB*, *wcbE*, and *wcbH* genes encode for proteins that demonstrate homology to different mannosyltransferases or glycosyltransferases from a variety of bacterial species (Table 3). Since type I O-PS is a homopolymer of mannoheptopyranosyl residues, it is likely that these genes are involved in the biosynthesis of this polysaccharide. The *wcbB* gene encodes for a protein with homology to a glycosyltransferase, WbpX, from *P. aeruginosa* as well as to mannosyltransferases from a variety of bacteria (45). The function of glycosyltransferases is to catalyze the sequential transfer of sugar residues from nucleotide precursors to the membrane-bound acceptor, undecaprenol phosphate-P-GlcNAc (58). The *wcbB* gene product is likely involved in the transfer of mannose residues in the synthesis of type I O-PS. This gene was determined to be required for the synthesis of type I O-PS based on two lines of evidence: the insertional inactivation of this gene using pSKM11 rendered the mutant strain, SR1015, negative for type I O-PS production; and a transposon mutant, SLR5 (Fig. 3), lacked type I O-PS due to the insertion of Tn5-OT182 in the *wcbB* gene. The *wcbE* and *wcbH* genes both predict proteins with homology to mannosyltransferases and are both required for the production of type I O-PS. This is supported by the fact that the insertion of Tn5-OT182 in both the *wcbH* and *wcbE* genes (SLR19 and SLR18, respectively) resulted in mutant strains lacking type I O-PS (Fig. 3). Furthermore, an internal fragment of the *wcbE* gene was cloned into pSKM11 and used to insertionaly inactivate this gene in *B. pseudomallei*. The resulting strain, SR1016, was found to lack type I O-PS and demonstrated attenuated virulence in the animal model (data not shown).

Another gene required for the production of type I O-PS is *wcbP*. This gene predicts a protein that shares homology to the YooP protein of *Mycobacterium tuberculosis* (Table 3). The YooP protein has been characterized as a putative oxidoreductase based on sequence comparisons (13). The function of the predicted *wcbP* gene product in *B. pseudomallei* is unclear; however, the insertion of Tn5-OT182 into this gene in the

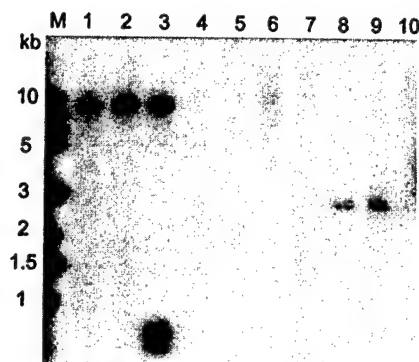


FIG. 4. Southern hybridization analysis of genomic DNA from *Burkholderia* spp. digested with *Sst*I. A 0.4-kb *Kpn*I-*Xho*I fragment from pDD1015 was used as a probe. Lane 1, *B. mallei* NCTC 10260; lane 2, *B. pseudomallei* 1026b; lane 3, *B. pseudomallei* SR1015; lane 4, *B. thailandensis* E264; lane 5, *B. vietnamiensis* LMG10929; lane 6, *B. cepacia* CEP509 (genomovar I); lane 7, *B. cepacia* K56-2 (genomovar III); lane 8, *B. stabilis* LMG14294; lane 9, *B. stabilis* LMG7000; lane 10, *B. multivorans* C5393.

mutant strain SLR13 rendered the organism negative for the production of type I O-PS.

The *yafJ* gene encodes a protein of 278 amino acids that demonstrates homology to the YafJ protein from *E. coli* (Table 3). The YafJ protein is a putative amidotransferase in these organisms (2). The *yggB* gene encodes a protein of 235 amino acids with homology to the YggB protein of *E. coli* (Table 3). The function of this protein is unclear, but it has been defined as a hypothetical 30.9-kDa protein in an intergenic region (2). The G+C contents of these genes are 65.7% for *yafJ* and 65.4% for *yggB*, which is higher than the rest of the polysaccharide cluster and consistent with the G+C content of the *B. pseudomallei* chromosome. Southern blot analysis using these genes as probes has demonstrated their presence in *B. thailandensis* (data not shown); therefore, it is unlikely that these genes are required for the production of type I O-PS (Fig. 3). An allelic exchange mutant containing a Tp cassette in the *yafJ* gene was constructed. The resulting strain, SR203::Tp, was found to be virulent in hamsters (data not shown).

The type I O-PS is also present in *B. mallei* and the *B. cepacia* complex but not in *B. thailandensis*. Southern blot analysis using a probe containing the A-T-rich glycosyltransferase fragment from pDD1015 confirmed that the fragment was present in *B. pseudomallei* 1026b and SR1015 but not in *B. thailandensis* (Fig. 4). *B. mallei* and the *B. cepacia* complex were also tested for the presence of this fragment. It was found that the probe hybridized to an *Sst*I fragment in *B. mallei* and the *B. stabilis* (formerly genomovar IV) strains LMG7000 and LMG14294. The *B. cepacia* complex has recently been divided into two genomovars (*B. cepacia* genomovar I and genomovar III) and three species (*B. multivorans*, *B. vietnamiensis*, and *B. stabilis*) (55, 56). None of the strains tested from *B. cepacia* genomovars I and III, *B. multivorans*, or *B. vietnamiensis* were found to contain this DNA fragment. Southern blot analysis was carried out on five other *B. stabilis* strains: CEP0717, CEP0467, J687, CEP0726, and LMG14291. All of the *B. stabilis* strains tested hybridized to the probe from pDD1015 (data not shown). The presence of type I O-PS was confirmed

in the *B. stabilis* strain LMG7000 by immunoelectron microscopy. As seen in Fig. 1D, *B. stabilis* LMG7000 showed reactivity to the type I O-PS antibodies, but lacked a uniform distribution of the polysaccharide on the cell surface, and therefore appears to produce less of this polysaccharide than *B. pseudomallei* 1026b.

Further Southern blot experiments were carried out to confirm the absence of the type I O-polysaccharide in *B. thailandensis*. *B. thailandensis* was hybridized with probes corresponding to a number of genes involved in the synthesis of the type I O-PS. The following genes were demonstrated by Southern hybridization to be present in *B. pseudomallei* but absent in *B. thailandensis*: *wcbA*, *wcbC*, *wcbD*, *wzm2*, *wzt2*, *wcbE*, *wcbF*, *wcbH*, *wcbK*, *gmhA*, and *wcbO* (data not shown).

DISCUSSION

Although melioidosis is less common outside of Southeast Asia and northern Australia, it may be underdiagnosed in other regions, and it poses a concern due to increased travel and military involvement in regions where the disease is endemic (14). Recently, our attention has been focused on the identification of genetic determinants that contribute to the pathogenesis of *B. pseudomallei* infections. To obtain virulence determinants unique to *B. pseudomallei*, we used subtractive hybridization between this organism and a related nonpathogenic organism, *B. thailandensis*.

Analysis of the subtractive hybridization library revealed that *B. pseudomallei* contains a number of DNA sequences that are not found in *B. thailandensis* (Table 2). One of the subtraction clones, pDD1015, demonstrated weak homology to a glycosyltransferase, WbpX, from *P. aeruginosa* (45). The insert from pDD1015 was cloned into a mobilizable suicide vector, pSKM11, for insertional inactivation of the glycosyltransferase gene in wild-type *B. pseudomallei*. The resulting strain, SR1015, was markedly less virulent than the parent strain in an animal model. This demonstrated that *B. pseudomallei* contains DNA sequences encoding for virulence determinants that are not found in *B. thailandensis* and that the glycosyltransferase gene may encode an important virulence determinant in *B. pseudomallei*. Using antibodies to type I O-PS, we determined that SR1015 harbored a mutation in a glycosyltransferase gene involved in the production of type I O-PS.

Sequence analysis of the DNA flanking the glycosyltransferase gene revealed the presence of at least 20 open reading frames involved in the synthesis and export of type I O-PS (Fig. 3; Table 3). The genes identified encode for proteins that are similar to proteins involved in the biosynthesis and export of capsular polysaccharides, particularly those involved in the production of group 3 capsular polysaccharides. Group 3 capsules include the *E. coli* K10 capsule and may also include the *H. influenzae* group b capsule and the capsule produced by *N. meningitidis* serogroup B (59). Group 3 capsules are always coexpressed with O serogroups, are not thermoregulated, are transported by an ABC-2 exporter system, and do not contain the *kpsU* and *kpsF* genes, and usually the gene clusters map near the *serA* locus (59). Thus far, no *serA* locus that is associated with the type I O-PS cluster has been identified, but this polysaccharide is coexpressed with O antigen and lacks the *kpsU* and *kpsF* genes, and genes encoding for a putative ABC-2

transporter have been identified. The genes involved in the production of group 3 capsules are organized into regions and are divergently transcribed. Regions 1 and 3 are generally conserved and contain genes involved in export of the polysaccharide. These regions flank region 2, which contains the biosynthetic genes and is not conserved between serotypes (44). The genetic organization of the type I O-PS is also similar to that of other capsule gene clusters in that the genes are organized into more than one transcriptional unit and appear to be divergently transcribed. However, the organization of the *B. pseudomallei* type I O-PS cluster differs in that the biosynthetic genes identified thus far are not organized into one biosynthetic region. *yafJ* and *yggB* are likely not involved in the production of type I O-PS since they have a high G+C content (62 to 65%), they are present in *B. thailandensis*, and a mutation in *yafJ* (SR203::Tp) did not reduce virulence in hamsters (data not shown). We are also currently constructing a mutant in the polyketide synthase gene that lies downstream of the *wcbP* gene in order to define this end of the type I O-PS cluster.

The polysaccharide with the structure -3)-2-O-acetyl-6-deoxy- β -D-manno-heptopyranose-(1- was originally isolated and characterized as an O-PS component of LPS in *B. pseudomallei* and was designated type I O-PS (41). However, our results suggest that this polysaccharide is a capsule rather than an O-PS moiety. The genes involved in the production of this capsule demonstrate strong homology to the genes involved in the production of capsular polysaccharides in many organisms, including *N. meningitidis*, *H. influenzae*, and *E. coli*. In addition, the export genes associated with this cluster are not associated with the previously characterized O-PS gene cluster (17). Western blot analysis of proteinase K cell extracts (Fig. 2) and silver staining (data not shown) have shown that this polysaccharide has a high molecular mass (200 kDa) and lacks the banding pattern seen with O-PS moieties. Studies by our laboratory have indicated that mutants in the production of the core oligosaccharide of the LPS are still capable of producing this polysaccharide (9). Based on the above criteria and the genetic similarity to group 3 capsules, we propose that this polysaccharide is a group 3 capsule.

Capsule production has been correlated with virulence in many bacteria, particularly those causing serious invasive infections of humans (4). Our studies have demonstrated that this capsule is critical for the virulence of *B. pseudomallei*. However, its specific role in infection has yet to be elucidated. A number of functions have been suggested for polysaccharide capsules: prevention of desiccation for transmission and survival, adherence for colonization, resistance to complement-mediated phagocytosis and complement-mediated killing, and resistance to specific host immunity due to a poor antibody response to the capsule (44). Preliminary studies have shown that type I O-PS is not involved in serum resistance. SR1015 was tested for resistance to killing by 30% normal human serum and was found to be resistant to killing (data not shown). Studies to define the role of the capsule in infection are under way.

Genomic DNAs from *B. mallei* NCTC 10260 and seven strains of *B. stabilis* were shown to hybridize to the glycosyltransferase probe from pDD1015. Immunoelectron microscopic analysis demonstrated that *B. stabilis* LMG7000 con-

tained this capsule (Fig. 1). Interestingly, *B. stabilis* LMG7000 was noted to produce less of this polysaccharide than *B. pseudomallei* 1026b and lacked a uniform distribution of the polysaccharide on the cell surface. The importance of the capsule in infection by *B. stabilis* has yet to be elucidated. The results of our study demonstrating the presence of this capsule in *B. stabilis* corresponds with its recent classification as a novel species (56). This capsule may be an additional tool to aid in the identification of *B. stabilis* strains.

Virulence genes of a number of pathogenic bacteria are located on pathogenicity islands (PAIs), regions on the bacterial chromosome that are present in the genome of pathogenic strains but rarely present in those of nonpathogenic strains. The PAIs may range in size from about 30 kb to 200 kb and often differ in G+C content from the remaining bacterial genome; the PAIs are often associated with the carriage of many virulence genes. These genetic units are often flanked by direct repeats and may be associated with tRNA genes or insertion sequence (IS) elements at their boundaries. They may also be associated with the presence of mobility genes, such as IS elements, integrases, transposases, and origins of plasmid replication. These DNA regions are considered to be unstable in that they may be subject to deletion with high frequency or undergo duplications and amplifications (23). A number of PAIs have been described for both gram-positive and gram-negative bacteria, and the application of subtraction hybridization has been used to successfully identify such genetic elements (23, 32). The subtractive hybridization that was carried out between *B. pseudomallei* and *B. thailandensis* led to the identification of a number of sequences that were found to be A-T rich compared to the rest of the *B. pseudomallei* chromosome. This, combined with the fact that insertional mutagenesis of the glycosyltransferase gene identified by this method resulted in an avirulent strain, suggests that we may have identified DNA sequences from a putative PAI and that the capsular polysaccharide gene cluster may be located on this island. It is possible that *B. pseudomallei*, *B. mallei*, and *B. stabilis* acquired DNA encoding for capsule as well as other potential, yet unidentified virulence factors by horizontal transfer recently in evolution. *B. pseudomallei* is known to contain IS elements that are present in *B. cepacia* but not in *B. thailandensis* (31). However, IS elements have not yet been identified in association with the capsule gene cluster. Further studies are under way to determine whether a PAI exists in these organisms and whether the capsule gene cluster is located on such a genetic element.

The identification of bacterial virulence genes has traditionally relied on empirical predictions of putative virulence determinants and inactivation of the genes encoding for these putative virulence determinants by any number of methods, followed by comparisons of virulence between mutant and wild-type infection models (19). Tools such as in vivo expression technology and differential fluorescence technology have been developed to facilitate the identification of expressed sequences under a given set of circumstances within a test host; however, these approaches do not necessarily lead to the identification of virulence determinants (53). The method for identification of virulence genes described herein should be applicable to a broad range of pathogenic bacteria. The combination of PCR-based subtractive hybridization, insertional

mutagenesis, and an animal infection model provides for the efficient detection of virulence genes. While we have applied the method to the pathogen *B. pseudomallei* in our current studies, it could be applied to any species and for which only a few prerequisites are in place. These prerequisites include related virulent and avirulent strains, suitable suicide vectors for insertional inactivation, and an infection model for differentiation of virulent and avirulent strains. The described method should lead to the identification of relevant virulence determinants for a number of bacterial species and further the understanding of molecular pathogenesis.

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Identification of a *Burkholderia mallei* polysaccharide gene cluster by subtractive hybridization and demonstration that the encoded capsule is an essential virulence determinant

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Little is known about the virulence factors of *Burkholderia mallei*, the etiologic agent of glanders. We employed subtractive hybridization to identify genetic determinants present in *B. mallei* but not in *Burkholderia thailandensis*, a non-pathogenic soil microbe. Three subtractive hybridization products were mapped to a genetic locus encoding proteins involved in the biosynthesis, export and translocation of a capsular polysaccharide. We identified an insertion sequence (IS407A) at one end of the capsule gene cluster and demonstrated that it was functional in *B. mallei*. Mutations were introduced in the *B. mallei* capsular gene cluster and the corresponding mutants were examined for their reactivity with antibodies raised against *Burkholderia pseudomallei* surface polysaccharides by immunoblotting and ELISA. Immunogold electron microscopy demonstrated the presence of a capsule on the surface of *B. mallei* ATCC 23344 (parental strain) but not on *B. mallei* DD3008 (capsule mutant) or *B. thailandensis*. Surprisingly, *B. thailandensis* also harboured a portion of the capsule gene cluster. ATCC 23344 was highly virulent in hamsters and mice, but DD3008 was avirulent in both animal models. The results presented here demonstrate that the capsular polysaccharide of *B. mallei* is required for production of disease in two animal models of glanders infection and is a major virulence factor.

Key words: glanders, melioidosis, pathogenesis and animal model.

Introduction

Glanders, one of the oldest diseases known to man, was first described by Aristotle [1]. It is a

disease of solipeds (horses, mules and donkeys) but incidental infections also occur in humans and carnivores [2–4]. Glanders was distributed worldwide until control measures were introduced in the early 20th century [5]. The disease has been eradicated from North America and western Europe but still persists in some

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South American, eastern European, African and Asian countries. Glanders in humans is almost always fatal without antibiotic intervention and it typically occurs in people who have occupations that put them in contact with glanders animals such as veterinarians, stablemen and farmers [3, 4, 6]. Laboratory-acquired glanders infections in researchers studying *Burkholderia mallei*, the etiologic agent of glanders, have also been reported [7, 8]. *B. mallei* is a critical biological agent (category B) because of its aerosol infectivity, severe course of infection and worldwide availability [4, 9, 10]. There are concerns that critical biological agents will be used in future acts of biological terrorism [9]. In fact, *B. mallei* was allegedly used as a biological weapon during World War I [11, 12]. No human or veterinary glanders vaccine is currently available.

B. mallei is an obligate parasite of horses, mules and donkeys and no other natural reservoir is known [4, 13]. The organism is a nonmotile Gram-negative bacillus that is closely related to *Burkholderia pseudomallei* and *Burkholderia thailandensis* [14, 15]. *B. pseudomallei* is a pathogenic microbe that causes the glanders-like disease melioidosis [16] and *B. thailandensis* is a non-pathogenic soil saprophyte [14]. Little is known about the molecular biology of the putative virulence determinants of *B. mallei*, as it is poorly characterized genetically. As a result, very little is known about the basic mechanism(s) of *B. mallei* pathogenesis. We are interested in identifying and characterizing those factors that are responsible for *B. mallei* pathogenesis at the molecular level. In this study, we performed subtractive hybridization between *B. mallei* and *B. thailandensis* and identified a genetic locus encoding a major *B. mallei* virulence determinant.

Results

Identification of a *B. mallei* polysaccharide gene cluster by subtractive hybridization

The goal of this study was to identify genetic determinants present in the pathogen *B. mallei*, but not in the non-pathogen *B. thailandensis* via subtractive hybridization. We sequenced 23 distinct plasmid inserts from a *B. mallei*-*B. thailandensis* subtractive hybridization library and selected three plasmids; pDD3006, pDD3008 and pDD3023 for further study. These plasmids were

selected because they contained inserts with relatively low G + C contents as compared to the rest of the *B. mallei* ATCC 23344 genome (68% G + C) [15]. The plasmids pDD3006, pDD3008 and pDD3023 contained DNA inserts with G + C contents of 50, 53 and 57%, respectively. The plasmid inserts were mapped to a genetic locus containing 28 genes, an insertion sequence and a gene remnant [Fig. 1(a)]. The majority of encoded proteins are similar to bacterial proteins involved in capsular polysaccharide biosynthesis, export and translocation [Table 1, Fig. 1(a)]. Genes involved in the biosynthesis of bacterial surface polysaccharides are commonly arranged in clusters and the genes in the cluster reported here have been assigned names based on the bacterial polysaccharide gene nomenclature (BPGN) scheme [17].

Molecular characterization of the *B. mallei* polysaccharide gene cluster

Bacterial polysaccharide biosynthetic gene clusters commonly encode products involved in three distinct processes: (1) biosynthesis of nucleotide sugars, or other components, needed for polysaccharide synthesis; (2) transfer of activated sugars from their nucleotide carriers to a growing carbohydrate chain; and (3) export and translocation of the resulting polysaccharide [17, 18]. The *B. mallei* polysaccharide gene cluster encodes at least 12 proteins involved in biosynthesis of nucleotide sugars, or other components, needed for polysaccharide synthesis [Table 1, Fig. 1(a)]. Included in this group is a protein that is homologous to eukaryotic heparan sulfate 6-sulfotransferases (WcbF) and four proteins that are similar to proteins involved in lipid biosynthesis, modification and transfer (WcbP, WcbR, WcbS and WcbT). WcbB, WcbE and WcbH are homologous to glycosyltransferases, proteins involved in the transfer of activated sugars from their nucleotide carriers to a growing carbohydrate chain [Table 1, Fig. 1(a)]. The proteins WcbA, WcbC, WcbD, Wzm, Wzt and WcbO are similar to proteins involved in export and translocation of group 3 capsular polysaccharides in *Escherichia coli*, *Haemophilus influenzae*, *Neisseria meningitidis*, *Campylobacter jejuni* and *Pasteurella multocida* [19–22]. Finally, there are two novel proteins (WcbG and WcbI) and four proteins that are homologous to proteins with unknown functions (DedA, YggB, YafJ and WcbQ) [Table 1, Fig.

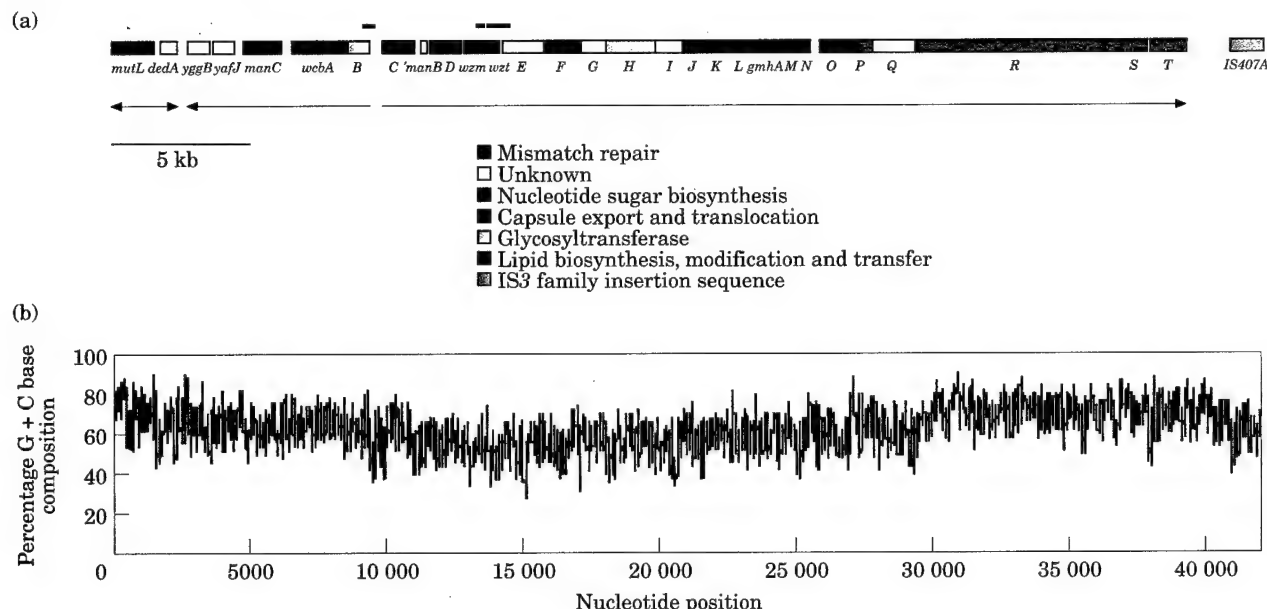


Figure 1. Genetic map and corresponding percentage G+C composition of the *B. mallei* polysaccharide gene cluster. (a) Genetic map of the polysaccharide gene cluster identified using three *B. mallei* subtractive hybridization products. The thin lines above the boxes represent the *B. mallei* subtractive hybridization products from pDD3006, pDD3008 and pDD3023 (Table 3). The boxes represent genes and an insertion sequence, and the arrows represent the transcriptional orientation of genes. The putative functions of the gene products are indicated. A scale bar, representing 5 kb, is shown. (b) The corresponding percentage G+C composition of the genetic locus shown in (a).

1(a)]. Taken together, these results suggest that the *B. mallei* polysaccharide gene cluster may be involved in the biosynthesis of a capsular polysaccharide.

We identified an insertion sequence of the IS3 family [23] 1.6 kb downstream of *wcbT* [Fig. 1(a)]. This IS element was 97% identical (1205 bp/1236 bp) to IS407 from *Burkholderia cepacia* [24] and was designated IS407A. IS407 and IS407A are 1236 bp in length and have 49 bp terminal inverted repeats. Like other members of the IS3 family, IS407A contains two partially overlapping reading frames, *orfA* and *orfB*, in the reading phases 0 and -1, respectively [23]. The functional transposase (OrfAB) of the IS3 family is generated by a programmed translational frameshifting event. A similar IS element was recently described in *B. pseudomallei*, but only a partial nucleotide sequence of that element was described [25, 26].

The 480 bp *wcbC*-*wcbD* intergenic region contains a 216 bp gene remnant that we have designated '*manB*' [Fig. 1(a)]. The gene remnant encodes a protein of 72 amino acids with similarity to the carboxy terminal portion of bacterial phosphomannomutases (Table 1). The

manB and *manC* genes are involved in the GDP-mannose biosynthesis pathway and often occurs in tandem [17]. It should be noted that the *B. mallei* polysaccharide gene cluster contains a copy of *manC* [Fig. 1(a)]. '*manB*' is non-functional as it does not contain start or stop codons and may represent the location of a previous genomic rearrangement event involving recombination, deletion or integration.

There is a decrease in the G+C content of the *B. mallei* polysaccharide gene cluster between nucleotide positions 11 000 and 30 000 as compared to the rest of the *B. mallei* genome (68% G+C) [Fig. 1(b)]. The G+C content of the region spanning nucleotide position 11 000 to 30 000 is 58%. In comparison, the G+C content between nucleotide positions 1-11 000 and 30 000-42 146 is 65 and 69%, respectively. Nucleotide sequences acquired via lateral gene transfer often contain a G+C content that differs from that of the rest of the recipient genome [27]. It is interesting to note that the start of the lowered G+C content at nucleotide position 11 000 corresponds to the location of the gene remnant '*manB*' (Fig. 1).

Table 1. Predicted *B. mallei* gene products presented in this study and similar proteins in the non-redundant sequence database

Protein	Size (aa/kDa)	Similar protein-Organism	Expect (E) value ^a	Putative function	Database accession number
MutL	—	MutL- <i>Neisseria meningitidis</i>	4e-90	Mismatch repair	AE002493
		MutL- <i>Salmonella typhimurium</i>	5e-69	Mismatch repair	A33588
DedA	226/24.8	DedA- <i>E. coli</i>	2e-57	Unknown	M68935
		DedA- <i>N. meningitidis</i>	3e-49	Unknown	AE002455
YggB	290/30.8	YggB- <i>Edwardsiella ictaluri</i>	4e-35	Unknown	AF037440
		YggB- <i>E. coli</i>	8e-28	Unknown	U28377
YafJ	278/31.5	Yn1191- <i>Candida albicans</i>	1e-32	Unknown	AJ250310
		Yn1191wp- <i>Saccharomyces cerevisiae</i>	2e-30	Unknown	Z71467
ManC	475/52.2	XanB- <i>Xanthomonas campestris</i>	1e-155	GDP-mannose pyrophosphorylase	M83231
		ManC- <i>E. coli</i>	1e-147	GDP-mannose pyrophosphorylase	U38473
WcbA	671/74.6	KpsC- <i>E. coli</i>	8e-89	Phospholipid substitution	X74567
		LipA- <i>N. meningitidis</i>	2e-81	Phospholipid substitution	AE002367
WcbB	280/31.7	WbpX- <i>Pseudomonas aeruginosa</i>	3e-29	Glycosyltransferase	AF010181
		MtfA- <i>Aquifex aeolicus</i>	8e-22	Mannosyltransferase A	AE000723
WcbC	387/40.9	CpxD- <i>Actinobacillus pleuropneumoniae</i>	4e-79	Capsule polysaccharide export	U36397
		CtrA- <i>N. meningitidis</i>	2e-78	Capsule polysaccharide export	AE002366
'ManB	72/8.2	ManB- <i>Yersinia pseudotuberculosis</i>	0.001	Phosphomannomutase	AJ251712
		manB- <i>Yersinia pestis</i>	0.001	Phosphomannomutase	AJ251713
WcbD	382/42.3	BexC- <i>Haemophilus influenzae</i>	4e-85	Capsule polysaccharide export	X54987
		CtrB- <i>N. meningitidis</i>	2e-78	Capsule polysaccharide export	M57677
Wzm	260/29.2	BexB- <i>H. influenzae</i>	3e-62	Capsule polysaccharide export	M33788
		CtrC- <i>N. meningitidis</i>	7e-62	Capsule polysaccharide export	M57677
Wzt	218/24.6	BexA- <i>H. influenzae</i>	3e-72	Capsule polysaccharide export	M19995
		CpxA- <i>A. pleuropneumoniae</i>	3e-71	Capsule polysaccharide export	AF143906
WcbE	507/56.8	MtfB- <i>A. aeolicus</i>	3e-19	Mannosyltransferase B	AE000693
		WbpX- <i>P. aeruginosa</i>	6e-14	Glycosyltransferase	AF010181
WcbF	440/50.2	BAA89249.1- <i>Mus musculus</i>	0.049	Heparan sulfate 6- sulfotransferase 3	AB024567
		BAA89248.1- <i>M. musculus</i>	0.11	Heparan sulfate 6- sulfotransferase 1	AB024566
WcbG	313/33.9	None			
WcbH	598/67.4	MtfA- <i>Archaeoglobus fulgidus</i>	4e-11	Mannosyltransferase A	AE001103
		SC6G10.05c- <i>Streptomyces coelicolor</i>	5e-04	Glycosyltransferase	AL049497
WcbI	311/35.9	None			
WcbJ	280/30.8	F6N23.17- <i>Arabidopsis thaliana</i>	1e-10	dTDP-6-deoxy-L-mannose- dehydrogenase	AF058919
		AAB86258.1- <i>Methanobacterium thermoautotrophicum</i>	2e-10	dTDP-4-dehydrorhamnose reductase	AE000933
WcbK	337/38.0	Gca- <i>Mycobacterium tuberculosis</i>	1e-48	Dehydrogenase	AL021926
		Gmd- <i>Yersinia pseudotuberculosis</i>	9e-37	GDP-mannose-4,6-dehydratase	AJ251712
WcbL	346/38.0	Cj1425c- <i>Campylobacter jejuni</i>	2e-85	Sugar kinase	AL139078
		Rv0115- <i>M. tuberculosis</i>	3e-81	Unknown	AL021926
GmhA	197/20.7	GmhA2- <i>C. jejuni</i>	1e-57	Phosphoheptose isomerase	AL139078
		GmhA-C- <i>C. jejuni</i>	3e-46	Phosphoheptose isomerase	AL139077

continued

Table 1. continued

Protein	Size (aa/kDa)	Similar protein-Organism	Expect (E) value ^a	Putative function	Database accession number
WcbM	230/24.9	Cj1423c-C. jejuni	4e-39	Sugar-phosphate nucleotidyltransferase	AL139078
		AAB86225.1-M. thermoautotrophicum	5e-23	mannose-1-phosphate guanylttransferase	AE000931
WcbN	189/20.3	YaeD-E. coli	4e-34	Unknown	U70214
		Cj1152c-C. jejuni	7e-34	Phosphatase	AL139077
WcbO	400/44.9	KpsS-E. coli	2e-62	Phospholipid substitution	X74567
		LipB-N. meningitidis	6e-47	Phospholipid substitution	Q05014
WcbP	262/27.8	YurA-Myxococcus xanthus	3e-21	Oxidoreductase	P25970
		HetN-Anabaena sp. PCC7120	3e-18	Ketoacyl reductase	L22883
WcbQ	511/56.2	RkpI-Sinorhizobium meliloti	4e-26	Unknown	Q52938
		Orf7-Streptococcus mutans	4e-15	Unknown	AB010970
WcbR	2546/ 267.6	LovF-Aspergillus terreus	1e-180	Type I polyketide synthase	AF141925
WcbS	305/33.1	EPOS C-Sorangium cellulosum	1e-180	Type I polyketide synthase	AF210843
		LpxC-H. influenzae	3e-36	UDP-3-O-[3-hydroxymyristoyl] N-acetylglucosamine deacetylase	U32794
		LpxC-E. coli	1e-34	UDP-3-O-[3-hydroxymyristoyl] N-acetylglucosamine deacetylase	X55034
WcbT	439/47.4	RkpG-S. meliloti	1e-105	Acyl-CoA transferase	X64131
		Kbl-E. coli	1e-57	2-amino-3-ketobutyrate coenzyme A ligase	X06690

^a A parameter that describes the number of hits one can "expect" to see just by chance when searching a database of a particular size.

Mutations in the polysaccharide gene cluster result in capsule-deficient mutants

Little is known about the genetics of *B. mallei*, and there are no published methods for constructing *B. mallei* mutants. In this study, we utilized a mobilizable suicide vector to construct merodiploid strains containing plasmid disruptions of genes in the polysaccharide gene cluster (see Experimental procedures). Since *B. mallei*, *B. pseudomallei* and *B. thailandensis* are closely related serologically [28-32], wild type and mutant strains were examined for their reactivity with rabbit antiserum directed against a *B. pseudomallei* surface polysaccharide with the structure -3)-2-O-acetyl-6-deoxy- β -D-manno-heptopyranose-(1-. Recent studies suggest that this structure represents a capsular polysaccharide rather than a glycoprotein [33, 34] or a LPS O-polysaccharide (O-PS) [35, 36]. *B. pseudomallei* 1026b and *B. mallei* ATCC 23344

demonstrated strong reactivity with the capsular antibodies in an ELISA, but *B. thailandensis* E264 demonstrated only background reactivity (Fig. 2). Seven *B. mallei* mutants (DD3105, DD3008, DD3101, DD3102, DD3103, DD3104 and DD3100) exhibited weak or partial reactivity with the capsular antibodies (Fig. 2). There was no difference in the reactivity of the mutants DD3108 and DD3107 and the parental strain ATCC 23344 in an ELISA (Fig. 2). The results demonstrate that *B. mallei* and *B. pseudomallei* produce an antigenically related polysaccharide capsule that is not present on *B. thailandensis*. The results also suggest that *manC*, *wcbB*, *wcbL*, *wcbM*, *wcbP*, *wcbQ* and *wcbR* are involved in the biosynthesis, export or translocation of the capsule in *B. mallei*. As the genes *yggB* and *yafJ* do not appear to be required for capsule production in *B. mallei* (Fig. 2), we suggest that *manC* defines the left side of the capsular gene cluster as depicted in Fig. 1(a).

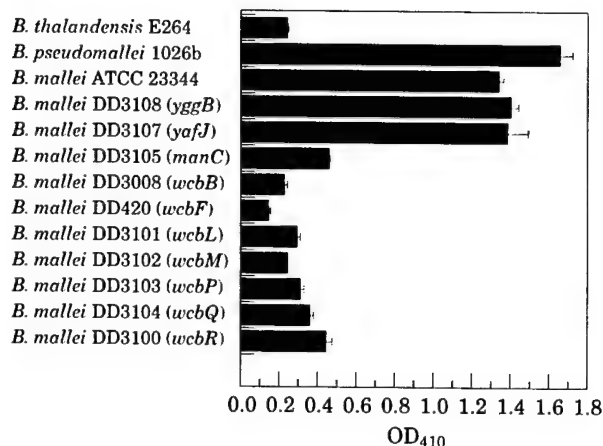


Figure 2. Whole-cell ELISA performed with polyclonal rabbit antiserum directed against the capsular polysaccharide of *B. pseudomallei*. OD₄₁₀, optical density at 410 nm. Disrupted genes are shown in parentheses following the strain names. The numerical values are the mean of one experiment performed in triplicate \pm SD.

IS407A is functional in *B. mallei*

In preliminary experiments, we serendipitously isolated a spontaneous capsule mutant after >10 laboratory passages of ATCC 23344. This mutant, termed DD420, did not react with the capsular antibodies in an ELISA (Fig. 2). We cloned and restriction mapped the capsule gene cluster in DD420 and identified a copy of IS407A at the 3' end of *wcbF* at nucleotide position 17 100. There was a 4 bp duplication of the sequence 5'-GCAG-3' flanking this copy of IS407A which is consistent with the generation of 4 bp direct target repeats by the IS3 family of IS elements [23]. These results indicate that IS407A is functional in *B. mallei* and that *wcbF* and/or a downstream gene(s) is essential for production of capsular polysaccharide. The relationship, if any, between laboratory passage and IS407A insertion in the capsule gene cluster is currently being investigated. However, we did not identify any IS407A insertions in the capsule gene cluster when we used animal passaged *B. mallei* for subsequent experiments.

Immunogold electron microscopy of *B. mallei* and *B. thailandensis*

We performed immunogold electron microscopy to see if we could identify a capsular structure on the surface of ATCC 23344, DD3008 and *B.*

thailandensis E264. The bacteria were reacted with polyclonal rabbit antiserum directed against the *B. pseudomallei* capsule, washed and reacted with a goat anti-rabbit IgG gold conjugate. ATCC 23344 reacted with the capsular antibodies and formed a thick (approximately 200 nm) and evenly distributed surface layer around the bacteria [Fig. 3(a)]. Similar results were obtained with *B. pseudomallei* 1026b [37]. DD3008, a representative capsule mutant, and *B. thailandensis* E264 did not react with the capsular antibodies [Fig. 3(b) and (c)]. These data directly demonstrate the presence of a capsule on the surface of ATCC 23344 that is immunologically cross-reactive with the *B. pseudomallei* capsular polysaccharide. The isogenic mutant DD3008 and *B. thailandensis* E264 do not produce this capsular polysaccharide.

B. mallei wild type and mutant strains produce LPS O-polysaccharides that are immunologically cross-reactive with those of *B. pseudomallei* and *B. thailandensis*

The LPS O-PS of *B. pseudomallei* and *B. thailandensis* consist of an unbranched heteropolymer with repeating D-glucose and L-talose units with the structure -3)- β -D-glucopyranose-(1-3)-6-deoxy- α -L-talopyranose-(1-[15, 35-37]. The chemical composition of the *B. mallei* LPS O-PS is currently unknown. We examined the cross-reactivity of whole cell lysates of *B. mallei*, *B. pseudomallei* and *B. thailandensis* with polyclonal rabbit serum raised against a *B. pseudomallei* polysaccharide-flagellin conjugate [38]. As expected, the polyclonal antiserum demonstrated reactivity with the capsule and LPS O-PS of *B. pseudomallei* 1026b in an immunoblot (Fig. 4, lane 3). The LPS O-PS exhibited a typical ladder-like appearance with apparent molecular weights ranging from <30-60 kDa (Fig. 4). The capsular polysaccharide migrated more slowly in the SDS-PAGE gel and was visualized by immunoblot as a smear with apparent molecular weights ranging from 75->100 kDa. *B. pseudomallei* SRM117, a LPS O-PS mutant [37], reacted with capsular antibodies but not with LPS O-PS antibodies (Fig. 4, lane 4). *B. thailandensis* E264, does not produce a polysaccharide capsule and only demonstrated reactivity with LPS O-PS antibodies (Fig. 4, lane 5). The E264 lysate also contained a band with an apparent molecular weight of 40 kDa that stained with a greater relative intensity. The

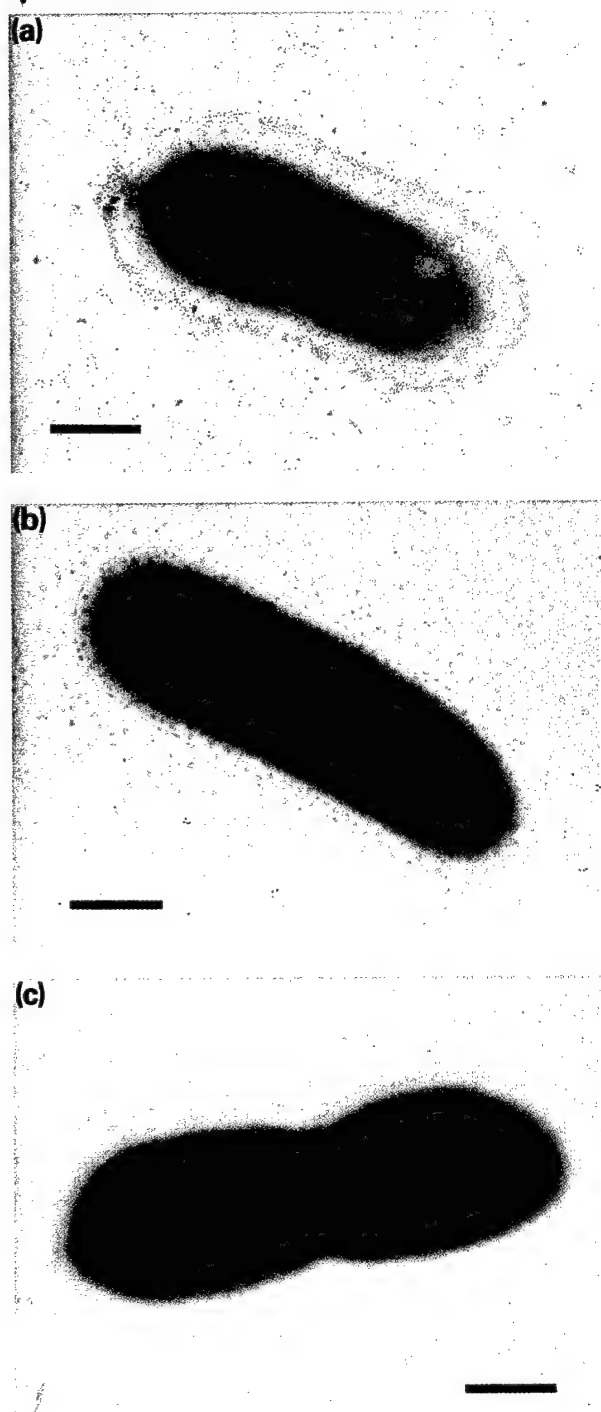


Figure 3. Immunogold electron microscopy of *B. mallei* ATCC 23344 (a), *B. mallei* DD3008 (b) and *B. thailandensis* E264 (c). Bars represent 500 nm.

identity of this product is currently unknown and it is not consistently present in our immunoblots. The capsule and LPS O-PS of ATCC 23344 demonstrated cross-reactivity with the

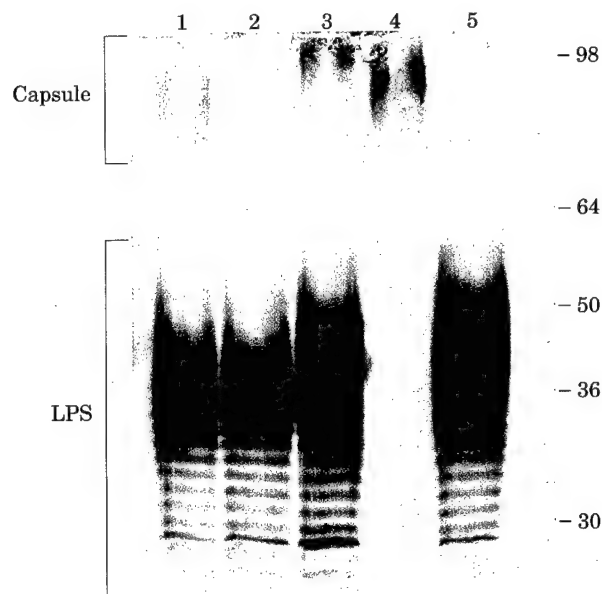


Figure 4. Immunoblot profiles of proteinase K-treated lysates of *B. mallei*, *B. pseudomallei* and *B. thailandensis*. The primary antibody used was polyclonal rabbit serum against a *B. pseudomallei* polysaccharide-flagellin conjugate [38]. The relative positions of capsule and LPS are indicated. Lanes: 1, *B. mallei* ATCC23344; 2, *B. mallei* DD3008; 3, *B. pseudomallei* 1026b; 4, *B. pseudomallei* SRM117; 5, *B. thailandensis*. The apparent molecular weights of the SeeBlue Pre-Stained Protein Standards (Novex) are presented in kDa.

polyclonal antiserum (Fig. 4, lane 1). DD3008 reacted with the LPS O-PS antibodies but not with the capsular antibodies (Fig. 4, lane 2). Similar results were obtained with the other *B. mallei* capsule mutants (data not shown). Taken together, the results demonstrate that *B. mallei*, *B. pseudomallei* and *B. thailandensis* produce LPS O-PS with similar banding patterns and immunological cross-reactivities and that *B. mallei* capsule mutants produce LPS O-PS.

B. mallei DD3008 is avirulent in two animal models of glanders infection

We compared the relative virulence of ATCC 23344 and DD3008 in Syrian hamsters by the intraperitoneal route and in BALB/c mice by the aerosol route. Hamsters are exquisitely sensitive to infection with virulent strains of *B. mallei* via the intraperitoneal route [39, 40]. The 50% lethal doses (LD₅₀s) of ATCC 23344 and DD3008 in the hamster model of infection were <10 and >10⁶ cfu, respectively. This represents a >10⁵-fold

difference in virulence between the wild type strain and the capsule mutant. In fact, hamsters infected with DD3008 remained clinically normal throughout the 5 day study. On day 6 of the infection, DD3008-infected animals were killed and their spleens and livers were cultured and tissue samples were fixed and processed for histopathological studies. The spleens and livers were sterile and no characteristic glanders lesions [39] were identified.

Mice were challenged by the aerosol route with *B. mallei* strains using a whole-body aerosol exposure apparatus [41]. The LD₅₀s of ATCC 23344 and DD3008 in the mouse model of infection were 913 and >10⁶ cfu, respectively. This represents a >10³-fold difference in virulence between the wild type strain and the capsule mutant. All of the DD3008-infected mice remained clinically normal throughout the 21 day study. As DD3008 displayed attenuated virulence in both hamsters and mice, we examined the possibility that it might be useful as an attenuated live vaccine strain. The DD3008-infected mice were rechallenged with approximately 20 LD₅₀s of ATCC 23344 on day 22 and deaths were recorded daily for 21 days. Eighty percent of the mice succumbed to infection after rechallenge with the wild type strain indicating that DD3008 will probably not be useful as an attenuated live vaccine strain. It should be noted that DD3008 grew normally in LB broth containing 4% glycerol.

The capsule gene cluster is also present in *B. thailandensis* but is truncated immediately downstream of *wcbC*_{Bt}.

We were interested in determining if *B. thailandensis* contained a capsule gene cluster similar to the one present in *B. mallei*. In preliminary experiments, the PCR primers used in the construction of *B. mallei* mutants were used to detect capsule genes in *B. thailandensis*. The genes *dedA*, *yafJ* and *manC* were detected in *B. thailandensis* via the PCR but the genes *wcbM*, *wcbP* and *wcbQ* were not (Table 2). As expected, all of the genes were detected in *B. mallei* (Table 2). In addition, we PCR-amplified a 1.6 kb product from *B. mallei* and *B. thailandensis* with the PCR primers DD14 and DD11, which are specific for *yggB* and *yafJ*, respectively (Table 2). This indicates that the chromosomal arrangement of *yggB* and *yafJ* is conserved between *B. mallei* and *B. thailandensis*. Taken together, these results suggest that the

left side of the capsule gene cluster, as depicted in Fig. 1(a), is present in *B. thailandensis*.

We cloned and sequenced 7.9 kb of the *B. thailandensis* capsule gene cluster and identified homologs of *B. mallei* *manC*, *wcbA*, *wcbB* and *wcbC* [Fig. 5(a)]. The arrangement of the genes were identical in the two species and the percentage nucleotide identities between the *manC*, *wcbA*, *wcbB* and *wcbC* alleles were 80, 55, 72 and 61%, respectively [Fig. 5(a)]. It is interesting to note that *B. thailandensis* contains a 373 bp region that corresponds to the 373 bp subtractive hybridization product from pDD3008 [Fig. 5(a)]. These sequences were only 54% identical and probably contained too many nucleotide differences to allow for efficient "subtraction" to occur using the subtractive hybridization conditions described here. As mentioned above, a gene remnant termed '*manB*' was identified immediately downstream of *wcbC*_{Bm} [Fig. 5(a)]. We determined the nucleotide sequence of 1.7 kb downstream of *wcbC*_{Bt} and did not identify a '*manB*' gene remnant or a *manB*-like gene. In fact, we did not identify any capsular polysaccharide genes in this region. Fig. 5(b) shows that the *B. mallei* and *B. thailandensis* capsule gene clusters diverge immediately following the *wcbC* stop codons. It should also be noted that there is a significant decrease in the G+C content of the *B. mallei* capsule gene cluster starting near the 3' end of *wcbC*_{Bm} [Fig. 1(b)]. These results suggest that *B. thailandensis* does not produce a polysaccharide capsule because the capsule gene cluster in this organism is truncated immediately downstream of *wcbC*_{Bt}.

Mapping the start site of transcription upstream of *wcbC* using 5' RACE

The nucleotide sequence of the capsule gene cluster indicates that *wcbB* and *wcbC* are divergently transcribed [Fig. 1(a)]. We performed 5' RACE (Rapid Amplification of cDNA Ends) in an attempt to identify transcriptional start sites and promoter elements in the 489 bp *wcbB*–*wcbC* intergenic region. The transcriptional start site of *wcbC* was mapped to a G residue 64 bp upstream of the *wcbC* ATG start codon (Fig. 6). We identified sequences that resemble *E. coli* σ^{70} –10 and –35 promoter elements immediately upstream of the *wcbC* start site of transcription (Fig. 6). The spacing between the *wcbC* –10 and –35 promoter elements was 20 bp and there was an "extended –10" motif (TG) [42] 1 bp

Table 2. Detection of capsule genes in *B. thailandensis* via PCR amplification

Gene(s)	PCR primers	Size of PCR product (bp)	PCR amplification results	
			<i>B. mallei</i>	<i>B. thailandensis</i>
<i>dedA</i>	DD15/DD16	407	+	+
<i>yafJ</i>	DD11/DD12	571	+	+
<i>manC</i>	DD9/DD10	772	+	+
<i>yggB-yafJ</i>	DD14/DD11	1598	+	+
<i>wcbM</i>	DD1/DD2	396	+	—
<i>wcbP</i>	DD5/DD6	398	+	—
<i>wcbQ</i>	DD7/DD8	650	+	—

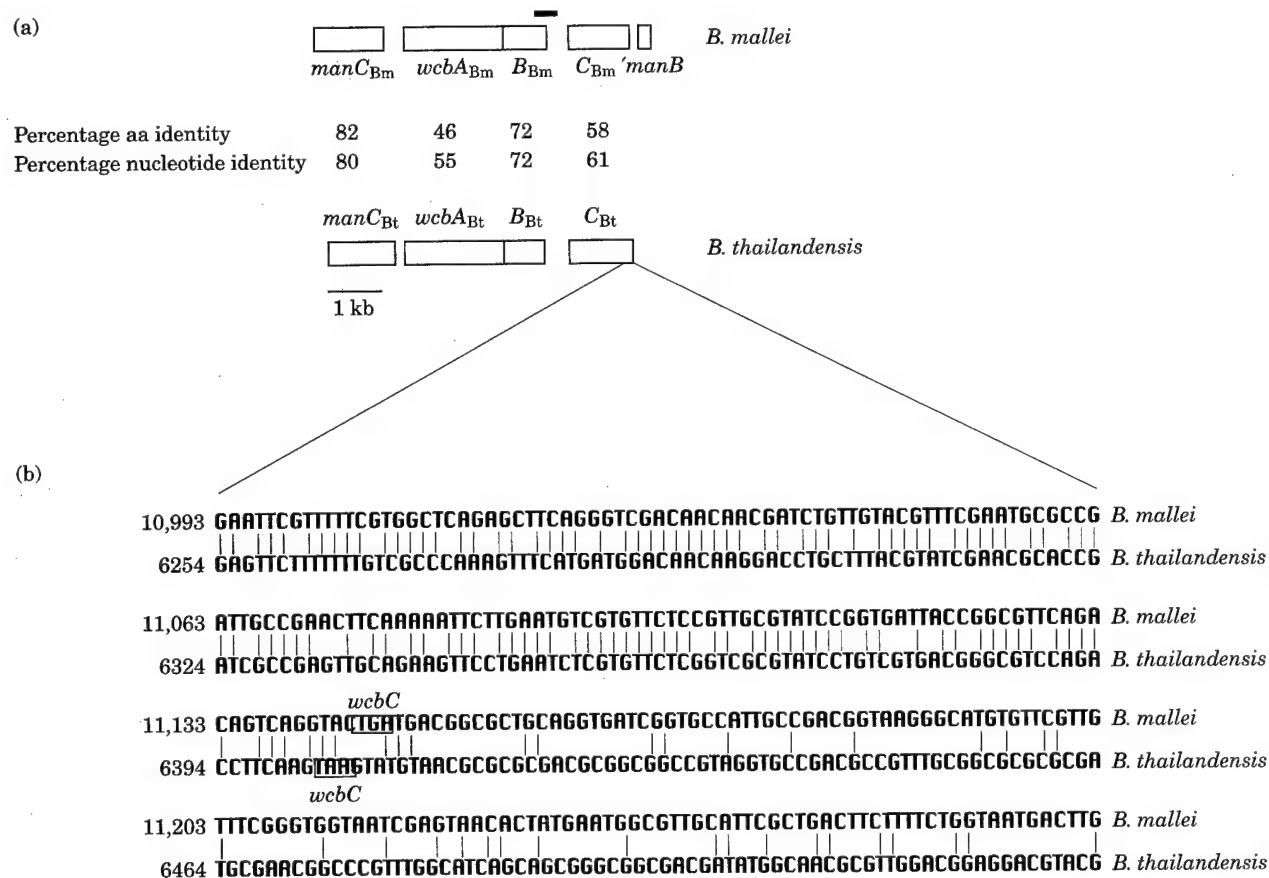


Figure 5. Comparison of the capsule gene clusters in *B. mallei* and *B. thailandensis*. (a) Comparison of *B. mallei* (Bm) and *B. thailandensis* (Bt) *manC*, *wcbA*, *wcbB* and *wcbC*. The boxes represent genes and the gene names are listed below (*B. mallei*) and above (*B. thailandensis*) the corresponding genes. The line above the *B. mallei* gene cluster represents the subtractive hybridization product from pDD3008. The percentage aa identity and percentage nucleotide identity between the *B. mallei* and *B. thailandensis* gene products and genes, respectively, are also shown. (b) Comparison of the nucleotide sequence at the 3' end and immediately downstream of *wcbCBm* and *wcbCBt*. Nucleotide sequence identities are represented by vertical bars between the corresponding nucleotides and the *wcbC* stop codons are boxed. The numbers to the left of the sequences correspond to the numbering in accession numbers AF285636 (*B. mallei*) and AF285634 (*B. thailandensis*).

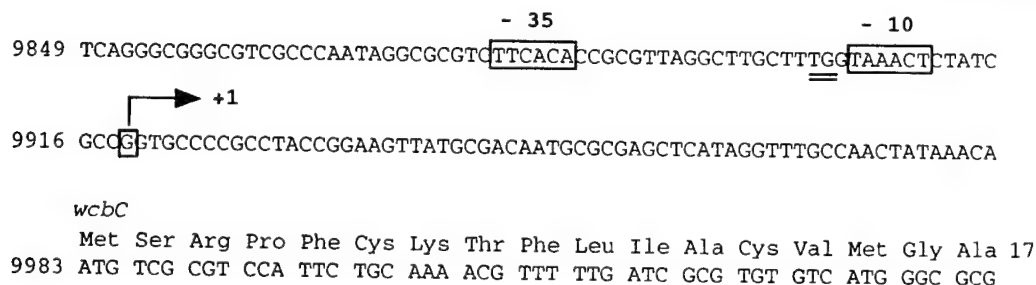


Figure 6. Mapping the transcriptional start site upstream of *wcbC* using 5' RACE. The transcriptional start site is boxed and indicated by an arrow and a +1. The -10 and -35 sequences of the σ^{70} -like promoter are boxed. The "extended -10" motif, immediately upstream of the -10 region, is double underlined. The first 17 codons of *wcbC* are shown and the corresponding amino acids are presented in three-letter code above the nucleotide sequence. The numbers to the left of the sequences correspond to the numbering in accession number AF285636.

upstream of the -10 promoter element (Fig. 6). Several attempts to identify the transcriptional start site upstream of *wcbB* using 5' RACE were unsuccessful.

Discussion

In this study, we performed subtractive hybridization between *B. mallei* and *B. thailandensis* and identified a gene cluster encoding a capsular polysaccharide in *B. mallei*. Popov et al. detected a capsular structure on the surface of *B. mallei* by ruthenium red staining and electron microscopy, but the chemical structure of the capsule was not determined [43, 44]. The *B. mallei* capsule described here exhibited immunological cross reactivity with antiserum raised against the *B. pseudomallei* capsule [Figs 3(a) and 4]. The *B. pseudomallei* capsule was previously thought to be a glycoprotein, termed surface antigen 8 [34], or a distinct LPS O-PS, termed type I O-PS [35, 36]. Recent work, however, indicates that the homopolymer of 2-O-acetyl-6-deoxy- β -D-mannoheptopyranose residues is probably a capsular polysaccharide [33]. There is no information on the chemical structure of the *B. mallei* capsule but the results presented here suggest that it is similar, and possibly identical, to the *B. pseudomallei* capsule.

While the pathogens *B. mallei* and *B. pseudomallei* produce a capsule, the closely related non-pathogenic species *B. thailandensis* does not [Figs 3(c) and 4] [14]. Surprisingly, *B. thailandensis* does possess a portion of the capsular gene cluster. A comparison of the *B. mallei* and *B. thailandensis* gene clusters revealed that the *B.*

thailandensis gene cluster was truncated immediately following the *wcbC_{Bt}* stop codon (Fig. 5). There were no polysaccharide genes identified in the 1.7 kb of DNA downstream of *wcbC_{Bt}* and gapped BLASTX and BLASTP searches [45] with this sequence did not identify any proteins with significant homology in the non-redundant nucleotide and protein databases. The DNA downstream of *wcbC_{Bm}* in *B. mallei* contains a gene remnant (*manB*) and a relatively low G+C content as compared to the rest of the *B. mallei* genome (Fig. 1). DNA acquired by lateral transfer often contains a G+C content that is markedly different from the recipient's DNA [27]. It is tempting to speculate that the capsule genes downstream of *wcbC_{Bm}* were acquired via lateral transfer after the divergence of *B. mallei* and *B. thailandensis* from a common progenitor. Alternatively, the complete capsule gene cluster may have been present in the common progenitor and subsequently lost in *B. thailandensis*. The *manB* gene remnant may represent the location where integration or deletion of laterally acquired DNA occurred.

Bacterial group 2 and 3 capsular polysaccharides share similarities in their biosynthesis and in the gene products required for this process [20, 22]. However, group 2 and group 3 gene clusters exhibit differences in gene arrangement and gene content. The *B. mallei* capsule gene cluster most closely resembles a group 3 gene cluster because of its gene arrangement and because it lacks the *kpsF* and *kpsU* homologues that are present in group 2 gene clusters. Group 3 capsule gene clusters are generally organized into three regions [20, 22]. Genes in region 1 and region 3 are involved in capsule export and translocation while genes in

region 2 are involved in biosynthesis of the capsular polysaccharide. The *B. mallei* gene cluster contains region 1 genes (*wbcC*, *wcbD*, *wzm* and *wzt*) but differs from other group 3 capsule gene clusters in that region 3 genes (*wcbA* and *wcbO*) are separated by >17 kb [Fig. 1(a)]. Furthermore, the region 2 genes are not clustered and are not flanked by region 1 and region 3 genes. It is likely that as more group 3 gene clusters are characterized more examples of differences in genetic organization will be revealed. In fact, the region 3 genes in the recently characterized *P. multocida* B:2 group 3 gene cluster are separated by >13 kb [46].

We identified an insertion sequence, IS407A, 1.6 kb downstream of *wcbT*. IS407A is a member of the IS3 family of insertion sequences [23] and is closely related to *B. cepacia* IS407 and an IS407-like element in *B. pseudomallei* [25, 26]. There are 1–4 copies of this element present in *B. pseudomallei* strains and 8–9 copies in *B. cepacia* strains [25, 26]. We detected >10 copies of IS407A in *B. mallei* ATCC 23344 by Southern blotting (data not shown). We also demonstrated that this element is functional in *B. mallei* as it inserted into *wcbF* in the spontaneous capsule mutant DD420. We also identified a *Bordetella parapertussis* IS1001-like element [47], termed ISBm1, in our *B. mallei*-*B. thailandensis* subtractive hybridization library (accession number AF285635). To our knowledge, these are the first IS element described in *B. mallei*. Insertion sequences are often present at the boundaries of pathogenicity islands and the *B. mallei* capsule gene cluster possesses most of the criteria of a pathogenicity island as defined by Hacker *et al.* (1997) [48]. IS407A may represent the right-hand boundary, as depicted in Fig. 1(a), of the capsule gene cluster as the 1.6 kb of DNA between *wcbT* and IS407A does not encode any proteins with significant homology to proteins in the non-redundant sequence databases. Further studies are in progress to determine if the IS407A element represents the right-hand boundary of the capsule gene cluster.

Shipovskaya *et al.* described *B. mallei* auxotrophic mutants generated by nitrosoguanidine mutagenesis [49], but there are no published reports describing the construction of genetically-defined mutations in this bacterium. We utilized the mobilizable suicide vector pGSV3 to clone the *B. mallei* capsule gene cluster by plasmid rescue and to construct mutants by plasmid disruption. We constructed nine mutants using the plasmid disruption technique

(Fig. 2). These mutants were instrumental in identifying the left-hand boundary of the capsule gene cluster (*manC*) and in identifying seven genes (*manC*, *wcbB*, *wcbL*, *wcbM*, *wcbP*, *wcbQ* and *wcbR*) involved in capsule biosynthesis, export or translocation [Figs 1(a) and 2]. Since it is possible that the mutations in these strains have polar effects on downstream genes, we are currently developing techniques and vectors for the construction of non-polar mutations in *B. mallei* via allelic exchange (gene replacement).

Previous reports have demonstrated that *B. mallei*, *B. pseudomallei* and *B. thailandensis* are closely related serologically [28–32]. The immunoreactive patterns of the LPS molecules from these species were nearly identical (Fig. 4), demonstrating that they produce antigenically related LPS O-PS. Thus, it is likely that the LPS O-PS are responsible, at least in part, for the serological cross-reactivity of these bacteria. *B. pseudomallei* and *B. thailandensis* produce a LPS O-PS that consists of an unbranched heteropolymer with repeating D-glucose and L-talose units [14, 28, 36]. This molecule is required for serum resistance in both *B. pseudomallei* and *B. thailandensis* [36]. Further studies are required to determine the exact chemical structure of the *B. mallei* LPS O-PS and examine its role in serum resistance.

We identified the start site of transcription upstream of *wcbC* using 5' RACE (Fig. 6). The *wcbC* promoter resembles an *E. coli* σ^{70} promoter in that it contains σ^{70} -like -10 and -35 elements [50, 51]. The optimal spacing between σ^{70} promoter elements in *E. coli* is 17 bp but there are 20 bp separating these elements in the *wcbC* promoter (Fig. 6). The principal sigma factor of *Helicobacter pylori*, σ^{80} , is similar to *E. coli* σ^{70} but exhibits differences in the amino terminus and in region 4.2 [52]. The optimal spacing of the -10 and -35 elements in *H. pylori* σ^{80} promoters is 21 bp [53]. There is currently no information on the principal sigma factor of *B. mallei* and no other promoters have been experimentally identified in this organism. It is possible that the principal sigma factor of *B. mallei* more closely resembles *H. pylori* σ^{80} rather than *E. coli* σ^{70} . It is also interesting to note that the *wcbC* promoter contains an "extended -10" motif (5'-TG-3') one base upstream of the -10 element (Fig. 6). *E. coli* contains a number of activator-independent σ^{70} promoters that contain the "extended -10" motif [42]. There is no requirement for a -35 element in these promoters as the *E. coli* RNA polymerase σ^{70} subunit makes an alternative

contact with the "extended -10" motif instead [54]. Thus, the spacing between the -10 and -35 elements in the *B. mallei* *wcbC* promoter may be of no consequence as the *wcbC* promoter may represent an "extended -10" promoter.

Polysaccharide capsules are highly hydrated polymers that mediate the interaction of bacteria with their immediate surroundings [55]. As a result, these surface structures often play integral roles in the interaction of pathogens with their hosts [56]. In this report, we demonstrated that the capsule-negative mutant DD3008 was avirulent in hamsters and mice. There was a >5 log difference in the LD₅₀ of ATCC 23344 and DD3008 in hamsters and a >3 log difference in mice. There were no deaths or signs of clinical illness in the animals challenged with DD3008, including those animals that received doses as high as 10⁶ cfu. The animal studies demonstrate that the capsular polysaccharide is a major virulence factor of *B. mallei*. The capsule may prevent efficient clearance of *B. mallei* by the host immune system but the specific role(s) of the capsule in the pathogenesis of glanders remains to be elucidated.

Materials and Methods

Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are described in Table 3. *E. coli* was grown at 37°C on LB agar (Lennox L agar) or in LB broth (Lennox L broth). *B. mallei*, *B. pseudomallei* and *B. thailandensis* were grown at 37°C on LB agar or in LB broth containing 4% glycerol. *B. mallei* ATCC 23344 was serially passaged three times in Syrian hamsters and a stock culture was maintained at -70°C by mixing an equal volume of broth culture and 40% glycerol. Unless stated otherwise, all experiments were conducted using animal passaged *B. mallei* ATCC 23344 with limited laboratory subculture. When appropriate, antibiotics were added at the following concentrations: 100 µg of ampicillin (Ap), 5 µg of gentamicin (Gm), 15 µg of polymyxin B (Pm), 25 µg of streptomycin (Sm), 25 µg of kanamycin (Km) and 15 µg of tetracycline (Tc)/ml for *E. coli* and *B. mallei* and 100 µg of Sm and 50 µg of Tc/ml for *B. pseudomallei* and *B. thailandensis*.

DNA manipulation

Restriction enzymes and T4 DNA ligase were purchased from Roche Molecular Biochemicals (Indianapolis, IN, U.S.A.) and were used according to the manufacturer's instructions. DNA fragments used in cloning procedures were excised from agarose gels and purified with a GeneClean III Kit (Bio 101, CA, U.S.A.). Chromosomal DNA was isolated from *B. mallei* by using a previously described protocol [57]. Plasmids were purified from overnight cultures by using Wizard Plus SV Minipreps (Promega, Madison, WI, U.S.A.).

Subtractive hybridization

Subtractive hybridization was performed using *B. mallei* ATCC 23344 genomic DNA as the "tester" and *B. thailandensis* E264 genomic DNA as the "driver". The protocol described in the CLONTECH PCR-Select Bacterial Genome Subtraction Kit User Manual was followed except that the hybridization temperature was raised from 63 to 73°C. The subtractive hybridization products were cloned into pCR2.1-TOPO and transformed into chemically competent XL10-Gold cells.

Cloning of the *B. mallei* polysaccharide gene cluster

The DNA insert from pDD3008 was released with *EcoRI* and cloned into the corresponding site in the Gm^R suicide vector pGSV3 (Table 3). The resulting plasmid, pGSV3008, was electroporated into *E. coli* S17-1 and the plasmid was conjugated to *B. mallei* ATCC 23344 as described elsewhere [58]. Gm^Rpm^R transconjugants were identified after 48-72 h incubation at 37°C. A Gm^Rpm^R transconjugant was selected and named DD3008. DNA flanking the site of pGSV3008 recombination in the DD3008 chromosome was isolated by plasmid rescue (self-cloning) [58] with *Bam*HI and *Not*I. The terminal 12 kb on the right side of the polysaccharide gene cluster, as depicted in Fig. 1, was cloned using a similar strategy to that described above but with the plasmid pGSV3104 and the strain DD3104 (Table 3). The *B. thailandensis* gene cluster described in this report was also cloned using a similar strategy with the suicide vector

Table 3. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics ^a	Reference
Strains		
<i>E. coli</i>		
TOP10	General cloning and blue/white screening	Invitrogen
XL10-Gold	General cloning and blue/white screening	Stratagene
S17-1	Mobilizing strain, transfer genes of RP4 integrated in chromosome; Sm ^R Pm ^S	[60]
SM10	Mobilizing strain, transfer genes of RP4 integrated in chromosome; Km ^R Sm ^S	[60]
<i>B. mallei</i>		
ATCC 23344	Type strain; isolated in 1944 from a human case of glanders; Pm ^R Gm ^S	[15]
DD3108	ATCC 23344::pGSV3108; Gm ^R	This study
DD3107	ATCC 23344::pGSV3107; Gm ^R	This study
DD3105	ATCC 23344::pGSV3105; Gm ^R	This study
DD3008	ATCC 23344::pGSV3008; Gm ^R	This study
DD420	ATCC 23344::derivative; <i>wcbF</i> ::IS407A	This study
DD3101	ATCC 23344::pDD3101; Gm ^R	This study
DD3102	ATCC 23344::pGSV3102; Gm ^R	This study
DD3103	ATCC 23344::pGSV3103; Gm ^R	This study
DD3104	ATCC 23344::pGSV3104; Gm ^R	This study
DD3100	ATCC 23344::pDD3100; Gm ^R	This study
<i>B. pseudomallei</i>		
1026b	Clinical isolate; Sm ^R Tc ^S	[58]
SRM117	1026b derivative; <i>whiI</i> ::Tn5-OT182; does not produce LPS O-PS; Tc ^R	[37]
<i>B. thailandensis</i>		
E264	Type strain; soil isolate; Sm ^R Tc ^S	[14]
DD3106	E264::pSKM3106; Tc ^R	This study
Plasmids		
pCR2.1-TOPO	3.9 kb TA cloning vector; pMB1 oriR; Km ^R Ap ^R	Invitrogen
pDD3006	pCR2.1-TOPO containing 597 bp subtractive hybridization product corresponding to nucleotide position 13 749–14 341 in <i>B. mallei</i> capsule cluster	This study
pDD3008	pCR2.1-TOPO containing 373 bp subtractive hybridization product corresponding to nucleotide position 9341–9713 in <i>B. mallei</i> capsule cluster	This study
pDD3023	pCR2.1-TOPO containing 175 bp subtractive hybridization product corresponding to nucleotide position 13 444–13 616 in <i>B. mallei</i> capsule cluster	This study
pSKM11	Positive selection cloning vector; IncP oriT; Cole1 ori; Ap ^R Tc ^S	[61]
pSKM3106	pSKM11 containing 772 bp PCR fragment internal to <i>manC_{Bt}</i> ; Ap ^R Tc ^R	This study
pDD3106H	14.4 kb <i>HindIII</i> fragment from DD3106 obtained by self-cloning; Ap ^R Tc ^R	This study
pDD3106B	11.1 kb <i>BamHI</i> fragment from DD3106 obtained by self-cloning; Ap ^R	This study
pTnMod-OGm'	Minitransposon vector; pMB1 oriR; RP4 oriT; Tn5 <i>tnp</i> ; Gm ^R	[62]
pGSV3	pTnMod-OGm' derivative lacking the 1.5 kb <i>BglIII tnp</i> fragment; mobilizable Gm ^R suicide vector	This study
pGSV3108	pGSV3 containing 541 bp PCR fragment internal to <i>yggB</i> ; Gm ^R	This study
pGSV3107	pGSV3 containing 571 bp PCR fragment internal to <i>yafJ</i> ; Gm ^R	This study
pGSV3105	pGSV3 containing 619 bp PCR fragment internal to <i>manC_{Bm}</i> ; Gm ^R	This study
pGSV3008	pGSV3 containing 379 bp <i>EcoRI</i> fragment from pDD3008; Gm ^R	This study
pDD3101	pGSV3 containing 605 bp <i>EcoRI</i> fragment internal to <i>wcbL</i> ; Gm ^R	This study
pGSV3102	pGSV3 containing 396 bp PCR fragment internal to <i>wcbM</i> ; Gm ^R	This study
pGSV3103	pGSV3 containing 398 bp PCR fragment internal to <i>wcbP</i> ; Gm ^R	This study
pGSV3104	pGSV3 containing 650 bp PCR fragment internal to <i>wcbQ</i> ; Gm ^R	This study
pGSV3100	pGSV3 containing 506 bp <i>EcoRI-NotI</i> fragment internal to <i>wcbR</i> ; Gm ^R	This study
pDD3008B	27 bp <i>BamHI</i> fragment from DD3008 obtained by self-cloning; Gm ^R	This study
pDD3008N	25.9 bp <i>NotI</i> fragment from DD3008 obtained by self-cloning; Gm ^R	This study
pDD3104R	15.2 bp <i>RcaI</i> fragment from DD3104 obtained by self-cloning; Gm ^R	This study

^a R, resistant; S, susceptible; Sm, streptomycin; Pm, polymyxin B; Km, kanamycin; Gm, gentamicin; Tc, tetracycline; Ap, ampicillin.

Table 4. Oligodeoxyribonucleotides used for PCR amplification of internal gene fragments

Primer	Sequence (5' to 3')	Positions	Gene
DD1	ATCCTCTTGACGCGTTTGAG	24 418–24 437	<i>wcbM</i>
DD2	AGCAACCTGCGTTGATCAAG	24 814–24 795	<i>wcbM</i>
DD5	TTTTGTCGCGAGCATTGACG	27 207–27 226	<i>wcbP</i>
DD6	TTGACGAACCCCGGCAAAAC	27 605–27 586	<i>wcbP</i>
DD7	ATTCTCGGTCACTTCGATCG	28 535–28 554	<i>wcbQ</i>
DD8	CTCGAATACATGCGACAACG	29 185–29 166	<i>wcbQ</i>
DD9	TCGATGCATTTCGTCGAGAAG	5723–5704	<i>manC</i>
DD10	GTGGACTGATTTTCGGTCAG	4952–4971	<i>manC</i>
DD11	ATCGATCAGAGCCTGCATTC	4464–4445	<i>yafJ</i>
DD12	AACGACGTGCTGTGAAAGAG	3893–3912	<i>yafJ</i>
DD13	TGTCGCACTATCTGGAATCG	3426–3407	<i>yggB</i>
DD14	CGCAATACACTTGCCAGTAG	2866–2885	<i>yggB</i>
DD15	GACGAATGAACGGGATGTTG	2322–2303	<i>dedA</i>
DD16	GCGATTCGCTGCTCTTCATC	1915–1934	<i>dedA</i>

pSKM11. The plasmid pSKM3106 was recombined into the *B. thailandensis* E264 chromosome and the DNA flanking the recombination site was isolated from DD3106 by self-cloning with *HindIII* and *BamHI*.

Construction of the *B. mallei* mutants

We utilized pGSV3 (Table 3) for constructing *B. mallei* mutants. Briefly, we PCR-amplified internal fragments of the genes to be inactivated and cloned them into pGSV3. The recombinant plasmids were electroporated into *E. coli* S17-1 and conjugated to *B. mallei* ATCC 23344 for 8 h as described elsewhere [58]. Gm^RPm^R transconjugants were identified after a 48–72 h incubation at 37°C. In order to confirm that transconjugants contained the desired mutations, mutated alleles were isolated by self-cloning [58] and sequenced. The genome of each merodiploid strain constructed by this technique contains two copies of the DNA insert separated by pGSV3 DNA (single crossover). The mutants were stable and no revertants were identified in the absence of Gm selection. Chromosomal DNA was PCR-amplified in a 100 µl reaction mix containing 1X *Taq* PCR Master Mix (Qiagen, Valencia, CA, U.S.A.) and 1 µM of each oligonucleotide primer. The thermal cycling parameters used for the PCR were 97°C for 5 min followed by 30 cycles of 97°C for 30 s, 53°C for 30 s and 72°C for 30 s. The nucleotide sequence of the oligodeoxyribonucleotide primers used in the PCR are presented in Table 4.

DNA sequencing and analysis

DNA sequencing was performed by ACGT, Inc. (Northbrook, IL, U.S.A.). DNA and protein sequences were analysed with GeneJockeyII software for the Macintosh and the University of Wisconsin Genetics Computer Group Package [59]. The gapped BLASTX and BLASTP programs were used to search the non-redundant sequence database for homologous proteins [45]. The percentage G+C base composition of the 42 146 bp polysaccharide gene cluster was determined using MacVector 6.5 software with a window size of 50.

Enzyme-linked immunosorbent assay (ELISA)

The wells of a round bottom microtitre plate were coated with approximately 5×10^6 bacteria in 100 µl of 0.05 M carbonate buffer pH 9.6, and the plate was incubated for 2 h at 37°C. The wells were washed with PBS plus 0.05% Tween-20 and blocked with a 3% solution of skim milk in PBS-Tween for 1 h at 37°C. The wells were washed and a 1:1000 dilution of a rabbit polyclonal antibody [37] directed against *B. pseudomallei* -3)-2-O-acetyl-6-deoxy-β-D-mannoheptopyranose-(1- was added, and the plate was incubated at 37°C for 1 h. The wells were washed and a 1:1000 dilution of a goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma, St. Louis, MO, U.S.A.) was added to each well. The plate was incubated for 1 h at 37°C, washed and developed with the pNPP Liquid Substrate

System (Sigma) for 30 min and the optical density at 410 nm (OD₄₁₀) was determined.

Immunoblot analysis

Overnight bacterial cultures (50 µl) were centrifuged, washed with PBS and resuspended in 50 µl of sample buffer (4% SDS, 10% glycerol, 5% β-mercaptoethanol and bromophenol blue in Tris buffer, pH 6.8). The samples were boiled for 10 min and treated with proteinase K (25 µg dissolved in 10 µl sample buffer) at 37°C for 1 h. The samples were boiled for 3 min and loaded onto a 12% SDS-polyacrylamide gel. Following electrophoresis, the gel was blotted to an Immuno-Blot PVDF membrane (BIO-RAD, Hercules, CA, U.S.A.) and an immunoassay was performed. The primary antibody, polyclonal rabbit serum raised against a *B. pseudomallei* polysaccharide-flagellin conjugate [38], was used at a 1:250 dilution. The secondary antibody, a goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma), was used at a 1:500 dilution. Alkaline phosphatase was detected with SIGMA FAST BCIP/NBT substrate solution.

Immunogold electron microscopy

The procedure for immunogold electron microscopy was described previously [37].

Animal studies

Female Syrian hamsters (five per group) were infected by the intraperitoneal route with 10¹, 10² and 10³ *B. mallei* ATCC 23344 and 10³, 10⁴, 10⁵ and 10⁶ *B. mallei* DD3008. Deaths were recorded daily for 5 days and the LD₅₀ values were determined. On day 6 the surviving animals from each group were killed and their spleens and livers were homogenized and cultured. Spleen and liver tissue samples were fixed and processed for histopathological studies as described previously [39].

Female BALB/c mice (10 per group) were challenged by aerosol using a whole-body aerosol exposure apparatus in a Class III safety cabinet in a biological safety level 3 containment facility [41]. While it is not possible to rule out ingestion of bacteria as a result of grooming,

histopathological observations suggest that infection initiates in the lung rather than the alimentary canal when using the whole-body aerosol exposure apparatus (D. L. Fritz, unpublished observations). Mice were exposed to aerosols of 92, 1598, 12 425, 124 250 and 461 500 cfu of *B. mallei* ATCC 23344 and 33,285, 9200, 165 600 and 766 667 cfu of *B. mallei* DD3008. Deaths were recorded daily for 21 days and the LD₅₀ values were calculated. Mice in the *B. mallei* DD3008 groups were rechallenged with approximately 20 LD₅₀s of *B. mallei* ATCC 23344 on day 22 and deaths were recorded daily for 21 days.

All animals used in this research project were cared for and used humanely according to the following policies: the U.S. Public Health Service Policy on Humane Care and Use of Animals [1996]; the Guide for the Care and Use of Laboratory Animals [1996]; and the U.S. Government Principles for Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training [1985]. All USAMRIID animal facilities and the animal program are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. All animal use was approved by the Institutional Animal Care and Use Committee and conducted in accordance with federal Animal Welfare Act regulations.

RNA isolation and 5' RACE

B. mallei ATCC 23344 was grown to early to middle exponential phase (approximately 5 h) and RNA was isolated using the Promega SV Total RNA Isolation System. 5' RACE was performed using the GIBCO BRL 5' RACE System for Rapid Amplification of cDNA Ends. The sequence of the oligodeoxyribonucleotide primer used in first strand cDNA synthesis was 5'-GGAATATTGACATTGC-3'. The sequence of the nested oligodeoxyribonucleotide primer used in PCR amplification of dC-tailed cDNA was 5'-CAGATGGACACCTGAATCG-3'. The 5' RACE amplification products were cloned into pCR2.1-TOPO and transformed into *E. coli* TOP10 (Table 3).

Nucleotide sequence accession numbers

The nucleotide sequences reported in this paper have been deposited in the GenBank database

under the accession numbers AF285636 (*B. mallei*) and AF285634 (*B. thailandensis*).

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Burkholderia thailandensis E125 Harbors a Temperate Bacteriophage Specific for *Burkholderia mallei*

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Burkholderia thailandensis is a nonpathogenic gram-negative bacillus that is closely related to *Burkholderia mallei* and *Burkholderia pseudomallei*. We found that *B. thailandensis* E125 spontaneously produced a bacteriophage, termed ϕ E125, which formed turbid plaques in top agar containing *B. mallei* ATCC 23344. We examined the host range of ϕ E125 and found that it formed plaques on *B. mallei* but not on any other bacterial species tested, including *B. thailandensis* and *B. pseudomallei*. Examination of the bacteriophage by transmission electron microscopy revealed an isometric head and a long noncontractile tail. *B. mallei* NCTC 120 and *B. mallei* DB110795 were resistant to infection with ϕ E125 and did not produce lipopolysaccharide (LPS) O antigen due to IS407A insertions in *whiE* and *whiG*, respectively. *whiE* was provided in *trans* on a broad-host-range plasmid to *B. mallei* NCTC 120, and it restored LPS O-antigen production and susceptibility to ϕ E125. The 53,373-bp ϕ E125 genome contained 70 genes, an IS3 family insertion sequence (ISBt3), and an attachment site (*attP*) encompassing the 3' end of a proline tRNA (UGG) gene. While the overall genetic organization of the ϕ E125 genome was similar to λ -like bacteriophages and prophages, it also possessed a novel cluster of putative replication and lysogeny genes. The ϕ E125 genome encoded an adenine and a cytosine methyltransferase, and purified bacteriophage DNA contained both N6-methyladenine and N4-methylcytosine. The results presented here demonstrate that ϕ E125 is a new member of the λ supergroup of *Siphoviridae* that may be useful as a diagnostic tool for *B. mallei*.

The disease glanders is caused by *Burkholderia mallei*, a host-adapted pathogen that does not persist in nature outside of its horse host (32). Glanders is a zoonosis, and humans whose occupations put them into close contact with infected animals can contract the disease. There have been no naturally occurring cases of glanders in North America in the last 60 years, but laboratory workers are still at risk of infection with *B. mallei* via cutaneous (68) and inhalational (31) routes. Human glanders has been described as a painful and loathsome disease from which few recover without antibiotic intervention (33, 51). There is little known about the virulence factors of this organism, but a recent report indicates that the capsular polysaccharide is essential for virulence in hamsters and mice (24).

Burkholderia pseudomallei is the etiologic agent of the glanders-like disease melioidosis (21). As the names suggest, *B. mallei* and *B. pseudomallei* are closely related species (19, 56, 59, 69). These β -Proteobacteria can now be directly compared at the genomic level because the *B. pseudomallei* K96243 genomic sequence is available at the Sanger Institute website (<http://www.sanger.ac.uk/>) and the *B. mallei* ATCC 23344 genomic sequence is available at the TIGR (The Institute for Genomic Research) website (<http://www.tigr.org/>). Preliminary BLAST (4) comparisons indicate that the genes conserved between these species are ~99% identical at the nucleotide level. This high level of nucleotide identity makes it challeng-

ing to use nucleic acid-based assays to discriminate between *B. mallei* and *B. pseudomallei* (6, 71).

There are legitimate concerns that *B. mallei* and *B. pseudomallei* may be misused as biological weapons (16, 46, 51, 60), and there is compelling evidence that *B. mallei* has already been used in this manner (3, 74). Diagnostic assays should be developed to discriminate between these microorganisms in the event that they are misused in the future. The use of a combination of diagnostic assays may be necessary to discriminate between these species, including nucleic acid-based assays, phenotypic assays (colony morphology, motility, and carbohydrate utilization), enzyme-linked immunosorbent assay, intact cell matrix-assisted laser desorption ionization–time of flight, and bacteriophage susceptibility.

In 1957 Smith and Cherry described eight lysogenic *B. pseudomallei* strains that produced bacteriophage that were more active on *B. mallei* than on *B. pseudomallei* (67). In fact, bacteriophage E attacked *B. mallei* strains exclusively. Manzenink et al. (45a) found that 91% of their *B. pseudomallei* strains were lysogenic and that three bacteriophages, PP19, PP23, and PP33, could be used in combination to identify *B. mallei*. Unfortunately, these *B. mallei*-specific bacteriophage were not further characterized and are not readily available. It is interesting that neither study identified bacteriophage production by *B. mallei* strains.

The purpose of this work was to identify and characterize a *B. mallei*-specific bacteriophage and make it available to the scientific community. *Burkholderia thailandensis* is a nonpathogenic soil saprophyte that has been described as *B. pseudomallei*-like (9, 10), and there are no published reports describing bacteriophage production by this species. *B. thailandensis*

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TABLE 1. Plasmids used in this study

Plasmid	Relevant characteristics ^a	Source or reference
pBluescript KS	General cloning vector; ColE1; Ap ^r	Stratagene
pDW1	pBluescript KS containing 1,068-bp <i>Hind</i> III fragment from ϕ E125	This study
pDW3.2	pBluescript KS containing 3,231-bp <i>Hind</i> III fragment from ϕ E125	This study
pDW4.4	pBluescript KS containing 4,351-bp <i>Hind</i> III fragment from ϕ E125	This study
pDW5.5	pBluescript KS containing 5,448-bp <i>Hind</i> III fragment from ϕ E125	This study
pDW7.5	pBluescript KS containing 7,325-bp <i>Hind</i> III fragment from ϕ E125	This study
pDW9.5	pBluescript KS containing 9,025-bp <i>Hind</i> III fragment from ϕ E125	This study
pDW11	pBluescript KS containing 9,942-bp <i>Hind</i> III fragment from ϕ E125	This study
pDW18	pBluescript KS containing 12,983-bp <i>Hind</i> III fragment from ϕ E125	This study
pSKM11	Positive selection cloning and suicide vector; IncP oriT; ColE1 ori; Ap ^r Tc ^r	50
pSKM3.2	pSKM11 containing 3,231-bp <i>Hind</i> III fragment from ϕ E125; Ap ^r Tc ^r	This study
pDD5003B	37.4-kb <i>Bam</i> HI fragment from DD5003 obtained by self-cloning; Ap ^r Tc ^r	This study
pCR2.1	3.9-kb TA cloning vector; pMB1 oriR; Km ^r Ap ^r	Invitrogen
pAM1	pCR2.1 containing ϕ E125 gene27 downstream of the <i>lac</i> promoter	This study
pCM1	pCR2.1 containing ϕ E125 gene56 downstream of the <i>lac</i> promoter	This study
pDD70	pCR2.1 containing 3.2-kb NCTC 120 <i>wbiE</i> ::IS407A PCR fragment	This study
pDD71	pCR2.1 containing 3.2-kb DB110795 <i>wbiG</i> ::IS407A PCR fragment	This study
pDD72	pCR2.1 containing ATCC 23344 <i>wbiE</i>	This study
pSPORT 1	General cloning vector; ColE1; Ap ^r	Life Technologies
pSPORT 8.1	pSPORT 1 containing 8.1-kb <i>Hind</i> III- <i>Eco</i> RI fragment from pDD5003B	This study
pBHR1	Mobilizable broad-host-range vector; Km ^r Cm ^r	MoBiTec
pBHR1- <i>wbiE</i>	pBHR1 containing 1.8-kb <i>Eco</i> RI fragment from pDD72; Km ^r Cm ^r	This study

^a Abbreviations: Ap, ampicillin; Tc, tetracycline; Km, kanamycin; Cm, chloramphenicol.

E125, isolated in 1991 from soil in northeastern Thailand (70), spontaneously produced a temperate bacteriophage (ϕ E125) that attacked *B. mallei* but not any other bacterial species examined. The gene order and modular organization of the ϕ E125 genome is reminiscent of lambdoid bacteriophages (11, 34), and it contains several interesting features, including an insertion sequence, two DNA methylase genes, and a novel cluster of putative replication and lysogeny genes. Bacteriophage ϕ E125 exhibits a B1 morphotype and therefore is a new member of the family *Siphoviridae* (phage with long noncontractile tails) (1, 2).

MATERIALS AND METHODS

Bacterial plasmids, strains, and growth conditions. The plasmids used in this study are described in Table 1. The *B. mallei* strains used in this study are listed in Table 2. The following *B. pseudomallei* strains were used in this study: 316c, NCTC 4845, 1026b, WRAIR 1188, USAMRU Malaysia 32, Pasteur 52237, STW 199-2, STW 176, STW 115-2, STW 152, STW 102-3, STW 35-1, K96243, 576a, 275, 295, 296, 503, 506, 112c, 238, 423, 465a, 776, 439a, 487, 644, 713, 730, E8, E12, E13, E24, E25, E40, E203, E210, E214, E215, E250, E272, E277, E279, E280, E283, E284, E300, E301, E302, and E304 (5, 20, 22, 25, 26, 66, 76). *B. thailandensis* strains E27, E30, E32, E96, E100, E105, E111, E120, E125, E132, E135, E202, E251, E253, E254, E255, E256, E257, E258, E260, E261, E263, E264, E266, E267, E275, E285, E286, E290, E293, E295, and E299 (10, 66, 76) were also utilized in this study. Other *Burkholderia* species used in this study include *B. cepacia* LMG 1222 (genomovar I) (44), *B. multivorans* C5568, *B. multivorans* LMG 18823 (44), *B. cepacia* LMG 18863 (genomovar III) (44), *B. cepacia* 715j (genomovar III) (47), *B. stabilis* LMG 07000, *B. vietnamiensis* LMG 16232 (44), *B. vietnamiensis* LMG 10929 (44), *B. gladioli* 2-72 (62), *B. gladioli* 2-75 (62), *B. gladioli* 4-54 (62), *B. gladioli* 5-62 (62), *B. uboniae* EY 3383 (77), *B. coccovenans* ATCC 33664, *B. pyrrocinia* ATCC 15958, *B. glathei* ATCC 29195, *B. caryophylli* Pc 102, *B. andropogonis* PA-133, *B. kururiensis* KP23 (79), *B. sacchari* IPT101 (8), *Burkholderia* sp. strain 2.2N (13), and *Burkholderia* sp. strain T-22-8A. *Ralstonia solanacearum* FC228, *R. solanacearum* FC229, *R. solanacearum* FC230, *Pandoraea apista* LMG 16407 (17), *Pandoraea norimbergensis* LMG 18379 (17), *Pandoraea phoenicis* LMG 18087 (17), *Pandoraea pulmonicola* LMG 18106 (17), *Stenotrophomonas maltophilia* XM16 (39), *S. maltophilia* XM47 (39), *Pseudomonas aeruginosa* PAO (30), *P. aeruginosa* PA14 (55), *Pseudomonas syringae* DC3000 (73), *Salmonella enterica* serovar Typhimurium SL1344 (29), *Serratia marcescens* H11, *Escherichia coli* TOP10 (Invitrogen), S17-

1 λ pir (65), HB101 (7), MC4100 (15), DH5 α (Gibco BRL), JM105 (78), E2348/69 (41), and DB24 (36) were also used in this study. *E. coli* was grown at 37°C on Luria-Bertani (LB) agar (Lennox) or in LB broth (Lennox). *P. syringae*, *B. andropogonis*, *Burkholderia* sp. strain 2.2N, *Burkholderia* sp. strain T-22-8A, *B. glathei*, and *B. caryophylli* were grown at 25°C on LB agar or in LB broth containing 4% glycerol. All other bacterial strains were grown at 37°C on LB agar or in LB broth containing 4% glycerol. When appropriate, antibiotics were added at the following concentrations: 100 μ g of ampicillin, 30 μ g of chloramphenicol, 25 μ g of kanamycin, and 15 μ g of tetracycline per ml for *B. thailandensis*. *B. mallei* DD3008 was grown in the presence of 5 μ g of gentamicin per ml, and *B. mallei* NCTC 120 (pBHR1) was grown in the presence of 15 μ g of polymyxin B and 5 μ g of kanamycin per ml.

Spontaneous bacteriophage production by lysogenic *B. thailandensis* strains and UV induction experiments. *B. thailandensis* strains E264, E275, E202, E125, and E251 were grown in LB broth for 18 h at 37°C with shaking (250 rpm). One hundred microliters of each saturated culture was used to inoculate two LB broth (3-ml) subcultures. One set of subcultures was incubated for 5 h under the same conditions. The other set of subcultures was incubated for 3 h, poured into sterile petri dishes in a class II biological safety cabinet, subjected to a hand-held UV light source (254 nm) for 20 s (25 cm above the sample), pipetted back into culture tubes, and incubated for an additional 2 h. Both sets of subcultures were briefly centrifuged to pellet the cells, and the supernatants were filter sterilized (0.45- μ m-pore-size filters). The samples were serially diluted in suspension medium (SM) (40), and the numbers of PFU were assessed by using *B. mallei* ATCC 23344 as the host strain as described below. Bacteriophage was considered to be induced if the titer increased twofold (or more) after exposure to UV light. If bacteriophage titers did not increase twofold, the bacteriophage was not considered to be induced by UV light.

Bacteriophage ϕ E125 propagation and DNA purification. The protocols followed for picking plaques, titrating bacteriophage stocks, and preparing plate lysates were the same as those used for bacteriophage λ (61), with a few minor modifications. Briefly, 0.1 ml of ϕ E125 and 0.1 ml of a saturated culture of *B. mallei* ATCC 23344 ($\sim 5 \times 10^8$ bacteria) were mixed and incubated at 25°C for 20 min, and 4.8 ml of molten LB top agar (0.7%) containing 4% glycerol was added. The mixture was immediately poured onto LB plates containing 4% glycerol and incubated overnight at 37°C. For preparation of plate lysate stocks, 5 ml of SM was added to the plate, and bacteriophage was eluted overnight at 4°C without shaking. SM was harvested, bacterial debris was separated by centrifugation, and the resulting supernatant was filter sterilized (0.45- μ m-pore-size filters) and stored at 4°C. Bacteriophage ϕ E125 DNA was purified from a plate culture lysate using the Wizard Lambda Preps DNA Purification System (Promega). The ϕ E125 lysogen BML10 was isolated from a single turbid plaque

TABLE 2. Bacteria used to examine the host range of bacteriophage ϕ E125

Bacterium	Relevant information	Plaque formation ^a
<i>Burkholderia mallei</i>		
NCTC 120	LPS O-antigen mutant; <i>wbiE</i> ::IS407A	—
NCTC 10248		+
NCTC 10229		+
NCTC 10260		+
NCTC 10247		+
NCTC 3708		+
NCTC 3709		+
ATCC 23344		+
ATCC 10399		+
ATCC 15310		+
DB110795	Laboratory-passaged ATCC 15310; LPS O-antigen mutant; <i>wbiG</i> ::IS407A	—
BML10	ATCC 23344 (ϕ E125)	—
DD3008	ATCC 23344::pGSV3008; capsule mutant	+
<i>Burkholderia pseudomallei</i>	50 strains	—
<i>Burkholderia thailandensis</i>	32 strains	—
<i>Burkholderia cepacia</i>	Genomovar I; 1 strain	—
<i>Burkholderia multivorans</i>	2 strains	—
<i>Burkholderia cepacia</i>	Genomovar III; 2 strains	—
<i>Burkholderia stabilis</i>	1 strain	—
<i>Burkholderia vietnamiensis</i>	2 strains	—
<i>Burkholderia gladioli</i>	4 strains	—
<i>Burkholderia uboniae</i>	1 strain	—
<i>Burkholderia cocovenans</i>	1 strain	—
<i>Burkholderia pyrocinia</i>	1 strain	—
<i>Burkholderia glathei</i>	1 strain	—
<i>Burkholderia caryophylli</i>	1 strain	—
<i>Burkholderia andropogonis</i>	1 strain	—
<i>Burkholderia kururiensis</i>	1 strain	—
<i>Burkholderia sacchari</i>	1 strain	—
<i>Burkholderia</i> spp.	2 strains	—
<i>Ralstonia solanacearum</i>	3 strains	—
<i>Pandoraea apista</i>	1 strain	—
<i>Pandoraea norimbergensis</i>	1 strain	—
<i>Pandoraea pnomenusa</i>	1 strain	—
<i>Pandoraea pulmonicola</i>	1 strain	—
<i>Stenotrophomonas maltophilia</i>	2 strains	—
<i>Pseudomonas aeruginosa</i>	2 strains	—
<i>Pseudomonas syringae</i>	1 strain	—
<i>Salmonella enterica</i> serovar Typhimurium	1 strain	—
<i>Serratia marcescens</i>	1 strain	—
<i>Escherichia coli</i>	8 strains	—

^a +, present; —, absent.

formed on ATCC 23344. The plaque was picked with a Pasteur pipette, transferred to a tube containing 3 ml of broth media, and incubated overnight. The saturated culture was spread onto solid media with an inoculating loop, and 10 isolated colonies were tested for their ability to form plaques with ϕ E125. All of the colonies were resistant to infection with ϕ E125, and one was selected and designated BML10.

ϕ E125 sensitivity testing. Approximately 10^2 PFU was added to a saturated bacterial culture and incubated at 25°C for 20 min, and 4.8 ml of molten LB top agar (0.7%) containing 4% glycerol was added. The mixture was immediately poured onto a LB plate containing 4% glycerol and incubated overnight at 25 or 37°C, depending on the bacterial species being tested. Bacteria were considered to be sensitive to ϕ E125 if they formed plaques under these conditions and resistant if they did not. It should be noted that the positive control, *B. mallei* ATCC 23344, formed plaques in the presence of ϕ E125 after incubation at 25 and 37°C. No bacterial species tested formed plaques in the absence of ϕ E125.

Negative staining of ϕ E125. Bacteriophage ϕ E125 was prepared from 20 ml of a plate culture lysate (see above), incubated at 37°C for 15 min with Nuclease Mixture (Promega), precipitated with Phage Precipitant (Promega), and resuspended in 1 ml of Phage Buffer (Promega). The bacteriophage solution (~100 μ l) was added to a strip of parafilm M (Sigma), and a formvar-coated nickel grid (400 mesh) was floated on the bacteriophage solution for 30 min at 25°C. Excess fluid was removed, and the grid was placed on a drop of 1% phosphotungstic acid, pH 6.6, for 2 min at 25°C. Excess fluid was removed, and the specimen was

examined on a Philips CM100 transmission electron microscope. Nickel grids were glow discharged on the day of use.

DNA manipulation and plasmid conjugation. Restriction enzymes and T4 DNA ligase were purchased from Roche Molecular Biochemicals and were used according to the manufacturer's instructions. DNA fragments used in cloning procedures were excised from agarose gels and purified with a GeneClean III Kit (Bio 101). Bacterial genomic DNA was prepared by using the Masterpure DNA Kit (Epicentre) for methylase dot blot assays and by a previously described protocol (75) for all other experiments. Plasmids were purified from overnight cultures using Wizard Plus SV Minipreps (Promega). The broad-host-range plasmids pBHR1 and pBHR1-*wbiE* were electroporated into *E. coli* S17- λ pir (12.25 kV/cm) and conjugated to *B. mallei* NCTC 120 for 8 h as described elsewhere (22). Similarly, the suicide vector pSKM3.2 was electroporated into *E. coli* S17- λ pir and conjugated to *B. thailandensis* E125 for 8 h as described elsewhere (22). The resulting strain, *B. thailandensis* DD5003, contained pSKM3.2 integrated into the ϕ E125 genome at the 3.2-kb *Hind*III fragment. Chromosomal DNA was isolated from DD5003 and digested with the restriction endonuclease *Bam*HI, and the bacteriophage attachment site and flanking bacterial DNA were obtained by self-cloning (22).

Immunoblot analysis. Fifty microliters of a saturated broth culture of *B. mallei* was subjected to centrifugation, and the bacterial pellet was washed with phosphate-buffered saline, pH 7.4. The sample was resuspended in 50 μ l of sample buffer (4% sodium dodecyl sulfate [SDS], 10% glycerol, 5% 2-mercaptoethanol,

0.005% bromphenol blue in Tris buffer, pH 6.8) and boiled for 10 min. The sample was treated with proteinase K (25 µg dissolved in 10 µl of sample buffer) and incubated at 37°C for 1 h. Forty microliters of sample was boiled for 5 min, loaded onto a 4% polyacrylamide stacking gel–12% polyacrylamide separating gel, and SDS-polyacrylamide gel electrophoresis (PAGE) was performed using 1× Tris-Glycine SDS Running buffer (Novex). The gel was blotted to Immobilon-PVDF Membrane (Bio-Rad) by using a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad) according to the manufacturer's instructions. The membrane was subjected to a blocking step (5% skim milk, 0.1% Tween 20) and was reacted with a 1:2,000 dilution of 3D11, a monoclonal mouse immunoglobulin G1 (IgG1) antibody that reacts with *B. mallei* lipopolysaccharide (LPS) O antigen (Research Diagnostics, Inc.). Following several washing steps with blocking buffer, the membrane was reacted with a 1:5,000 dilution of peroxidase-labeled goat antibody to mouse IgG (Kirkgaard & Perry Laboratories, Inc. [KPL]). Finally, it was washed three times with blocking buffer and once with PBS (pH 7.4) and then incubated with TMB Membrane Peroxidase Substrate (KPL).

DNA sequencing and analysis. DNA sequencing was performed at ACGT, Inc. (Northbrook, Ill.) and at LMT Sequencing Lab (Frederick, Md.). Most ϕ E125 genes were identified by using GeneMark.hmm (43), whereas others were identified by visual inspection, guided by BLAST (4) results. DNA and protein sequences were analyzed with GeneJockeyII and MacVector 7.1 software for the Macintosh. The gapped BLASTX and BLASTP programs were used to search the nonredundant sequence database for homologous proteins (4). In order to determine the nucleotide sequence of the ϕ E125 *cos* sites, we sequenced the ends of ϕ E125 DNA directly by using the following primers: COS4, 5'-AATCCGGCTCGTCCTTATTC-3' and COS10, 5'-GTTGCGGTGACGTGGTGGTG-3'. The nucleotide sequences obtained contained a gap relative to the ligated ϕ E125 ends on pDW9.5, which corresponded to unsequenceable 3' ends (64).

PCR amplifications. PCR products were sized by using agarose electrophoresis and cloned using the pCR2.1 TOPO TA cloning kit (Invitrogen) and chemically competent *E. coli* TOP10 (Invitrogen). PCR amplifications were performed with a final reaction volume of 100 µl and contained 1× Taq PCR Master Mix (Qiagen), 1 µM oligodeoxynucleotide primers, and approximately 200 ng of genomic DNA. PCR mixtures were transferred to a PTC-150 MiniCycler with a Hot Bonnet accessory (MJ Research) and heated to 97°C for 5 min. This was followed by 30 cycles of a three-temperature cycling protocol (97°C for 30 s, 55°C for 30 s, and 72°C for 2 min) and one cycle at 72°C for 10 min. The eight oligodeoxynucleotide primer pairs used in the PCR amplification of the LPS O-antigen gene cluster were as follows: 1-1, 5'-CGAGTTCACGGTATCACAA G-3', and 1-2, 5'-GTTGTCGTAGAGTACAGCC-3'; 2-1, 5'-GGCTGTACTTCTACGACAAC-3', and 2-2, 5'-GCATCAGCAGCGGATTGAAG-3'; 3-1, 5'-CTTCAATCCGCTGCTGATGC-3', and 3-2, 5'-GAATGCGACTTCAACAAC AC-3'; 4-1, 5'-GTGTTGTTGAAGTCGATTC-3', and 4-2, 5'-CATAAACGT TCTGCAGACGC-3'; 5-1, 5'-GCGTCTGCAGAACGTTTATG-3', and 5-2, 5'-GATTGCTGCAAAATAGCGTG-3'; 6-1, 5'-CACGCTATTGACGAAAT C-3', and 6-2, 5'-CGAAGATATCGAGCCAGTGC-3'; 7-1, 5'-GCAGTGGCTC GATATCTTCG-3', and 7-2, 5'-CCGAAGCGGTTGAAGAAGTG-3'; 8-1A, 5'-CTGGAATGGCTATGAGCAG-3', and 8-2A, 5'-AAATGCTCGCGTCATG TTGC-3'.

In order to determine the order and orientation of the *Hind*III fragments in the intact ϕ E125 genome, outward-oriented primers specific for the ends of each *Hind*III fragment (except the 1,068-bp fragment) were synthesized and PCR was performed with ϕ E125 genomic DNA and all possible primer combinations. We reasoned that two *Hind*III fragments were adjacent if we obtained a PCR product with primer pairs specific for the corresponding ends of those fragments. All PCR products were cloned and sequenced to confirm the PCR results. For these PCRs, and all of the PCR experiments mentioned below, the conditions mentioned above were used, with the following exception: we used 72°C for 30 s instead of 72°C for 2 min in the three-temperature cycling protocol. The 14 oligodeoxynucleotide primers used in this analysis were as follows: 3.2F, 5'-AGACGATCAAGCAACACAG-3'; 3.2R, 5'-TCGAAGCGCCAAATAAA ACGC-3'; 4.4F, 5'-CAAGCTCTCTAGCTTCTCG-3'; 4.4R, 5'-ACCAGCGG CCATACATTATG-3'; 5.5F, 5'-GGTCTCCGGATCGTAATTGT-3'; 5.5R, 5'-TCGTGCGTCAGTTCAATGG-3'; 7.5F, 5'-CCAGATCCGAATACGCCA C-3'; 7.5R, 5'-ATAACGCGCTTTGTGCGATCG-3'; 9.5F, 5'-GAGTGAAGCCA TCGAAGATC-3'; 9.5R, 5'-ACGGAAGGAGCATGTCATC-3'; 11F, 5'-TCA TCGACGAGGAACCTTAC-3'; 11R, 5'-AATGATGGTCAGCAGCAACG-3'; 18F-2, 5'-TCAAGGTAGAACAGCGTGTG-3'; 18R, 5'-GCTCCTTGTCCTA GTAGATG-3'.

PCR was performed with genomic DNAs from *B. mallei* ATCC 23344, *B. mallei* BML10, ϕ E125, and the primers Pro (5'-TATACCGACCGAATTGG-3') and Int (5'-TATGACGTGAAGCACTC-3') to determine if ϕ E125 inte-

grated into the proline tRNA (UGG) gene in *B. mallei*. We obtained a single PCR product of the expected size (550 bp) with *B. mallei* BML10 DNA. This product was cloned, and its nucleotide sequence was determined. No PCR products were obtained when genomic DNAs from *B. mallei* ATCC 23344 or ϕ E125 were used in the PCR.

Genomic DNA from ϕ E125 was used for PCR amplification of gene27 with the following primers: AM-UP, 5'-CAAGTTTAAAAACGGCTTTCAC-3', and AM-DOWN, 5'-CAGCCAATCGATCAGAACAG-3'. The resulting PCR product was cloned, sequenced, and designated pAM1 (Table 1). Similarly, gene56 was amplified by PCR using ϕ E125 genomic DNA and the following primers: CM-UP, 5'-CACAGGTGCTGTTCATCTC-3', and CM-DOWN, 5'-CTCAC ATGACCTCCAAAACG-3'. The resulting PCR product was cloned, sequenced, and designated pCM1 (Table 1).

Dot blot assay for DNA methyltransferase activity. The plasmids pCR2.1, pAM1, and pCM1 (Table 1) were electroporated into *E. coli* DB24, a strain that lacks all endogenous DNA methylation (36). The transformants were grown overnight in the presence of 1 mM IPTG (isopropyl- β -D-thiogalactopyranoside), and genomic DNA was isolated as described above. Genomic DNA preparations were diluted in Tris-EDTA buffer (10 mM Tris-Cl [pH 7.4], 1 mM EDTA [pH 8.0]) to yield stocks of 150, 50, and 15 ng/µl, and 3-µl aliquots of each were spotted onto a BA85 nitrocellulose filter (Schleicher & Schuell). Methylase activity was assessed by using rabbit primary antibodies that react specifically with DNA containing N6-methyladenine (m6A) or N4-methylcytosine (m4C) in a dot blot assay, as described previously (36). The secondary antibody was a peroxidase-labeled goat anti-rabbit IgG (H + L) conjugate (KPL). Primary and secondary antibodies were used at dilutions of 1:50,000 and 1:1,000, respectively. Detection was accomplished by using the luminol system (Amersham/Pharmacia), and exposures were made to hyperfilm-ECL (Amersham/Pharmacia). The film images were digitally captured using a UMAX flatbed scanner (S900) and Adobe Photodeluxe (version 1.1) software for the PowerMac.

GenBank and American Type Culture Collection (ATCC) accession numbers. The nucleotide sequences reported in this paper were deposited in the GenBank database under the accession numbers AF447491 (ϕ E125 genome) and AY063741 (*B. thailandensis* bacteriophage attachment site). ϕ E125 was deposited in the ATCC bacteriophage collection and was assigned the accession number ATCC 23344-B1.

RESULTS

***B. thailandensis* strains spontaneously produce bacteriophage that infect *B. mallei*.** Five strains of *B. thailandensis* (E125, E202, E251, E264, and E275) were examined for the production of *B. mallei*-specific bacteriophage. All of the strains, with the exception of E251, spontaneously produced bacteriophage that formed plaques with diameters of 1.5 to 2 mm on *B. mallei* ATCC 23344. Strain E264 produced two bacteriophages that formed distinct plaques, one turbid and one clear. Strains E125, E202, and E275 each produced a bacteriophage that formed turbid plaques. Bacteriophage production was increased 2-fold (E264 and E275), 6-fold (E125), and 55-fold (E202) by brief exposure to UV light. The clear plaque bacteriophage from E264 was not induced, and UV light did not induce bacteriophage production by E251. We examined the host range of all five *B. thailandensis* bacteriophages on 10 strains of *B. mallei* and 13 strains of *B. pseudomallei* and found that the temperate bacteriophages produced by E264, E202, and E275 formed plaques on 9 of 10 *B. mallei* strains and on 3 of 13 *B. pseudomallei* strains. Since these bacteriophages were not specific for *B. mallei*, they were not further characterized. The clear plaque bacteriophage produced by E264 (LPE264) and the temperate bacteriophage produced by E125 (ϕ E125) formed plaques on 8 of 10 and 9 of 10 *B. mallei* strains, respectively. Neither bacteriophage formed plaques on *B. pseudomallei* or on *B. mallei* NCTC 120. Typical yields of plate lysate stocks of LPE264 were 10⁵ PFU/ml, and yields of ϕ E125 were 10⁸ PFU/ml. Bacteriophage

LPE264 was not further characterized in this study due to its low yield and its inability to form plaques on *B. mallei* NCTC 3709. Taken together, these results indicate that lysogenic *B. thailandensis* strains exist in nature and that the bacteriophage they harbor are spontaneously produced and infect *B. mallei*.

Bacteriophage ϕ E125 is *B. mallei* specific. The host range of ϕ E125 was examined with 139 bacterial strains, including 13 strains of *B. mallei*, 50 strains of *B. pseudomallei*, and 32 strains of *B. thailandensis* (Table 2). Bacteriophage ϕ E125 formed plaques on 9 of 10 *B. mallei* strains obtained from NCTC and ATCC. It also formed plaques on DD3008, a capsule-deficient mutant derived from ATCC 23344 (24). Three *B. mallei* strains were resistant to plaque formation by ϕ E125, NCTC 120, DB110795 (a laboratory-passaged derivative of ATCC 15310), and BML10 (ATCC 23344 harboring the ϕ E125 prophage).

ϕ E125 did not form plaques on any of the *B. pseudomallei* or *B. thailandensis* strains used in this study (Table 2). It should be noted that the *B. pseudomallei* strains employed in this study were from a variety of sources; 15 clinical isolates, 30 Thai soil isolates, and 5 Australian soil isolates. Similarly, the *B. thailandensis* strains were isolated in northeastern Thailand (15 strains) and central Thailand (17 strains).

Finally, ϕ E125 plaque formation was evaluated with 15 additional species of *Burkholderia*, 4 species of *Pandoraea*, 2 species of *Pseudomonas*, *Ralstonia solanacearum*, *Stenotrophomonas maltophilia*, *S. enterica* serovar Typhimurium, *Serratia marcescens*, and *E. coli*. None of these bacteria formed plaques with bacteriophage ϕ E125 (Table 2). These results demonstrate that bacteriophage ϕ E125 forms plaques only on *B. mallei* strains, that ϕ E125-resistant *B. mallei* strains exist, and that the capsular polysaccharide (24) is not required for plaque formation by ϕ E125.

ϕ E125 is a new member of the family Siphoviridae. Bacteriophage may be tailed, cubic, filamentous, or pleomorphic and can be classified by morphotype and host genus (2). Numerous negatively stained bacteriophage were examined, and a representative image of ϕ E125 is shown in Fig. 1. ϕ E125 possessed an isometric head of 63 nm in diameter and a long noncontractile tail of 203 nm in length and 8 nm in diameter. Based on its B1 morphotype, ϕ E125 can be classified as a member of the order Caudovirales and the family Siphoviridae (1, 2). To our knowledge, this is the first bacteriophage of the Siphoviridae family described as being harbored by the host genus *Burkholderia* (2).

LPS O antigen is required for plaque formation by ϕ E125. Of the 10 *B. mallei* strains obtained from NCTC and ATCC, only NCTC 120 was resistant to plaque formation by ϕ E125 (Table 2). We hypothesized that resistance was due to the absence of a surface receptor for ϕ E125 on NCTC 120. The result obtained with DD3008 demonstrated that the capsular polysaccharide was not the ϕ E125 receptor (Table 2). We next performed an immunoblot on whole-cell lysates of the NCTC and ATCC strains with a commercially available monoclonal antibody (3D11) that reacts with *B. mallei* LPS O antigen (Fig. 2A). All of the NCTC and ATCC *B. mallei* strains, with the exception of NCTC 120, demonstrated a typical ladder LPS appearance after immunostaining with 3D11 (Fig. 2A). The laboratory-passaged derivative of ATCC 15310, termed DB110795, also does not form plaques with ϕ E125 (Table 2). We performed an immunoblot on a whole cell lysate of



FIG. 1. Transmission electron micrograph of bacteriophage ϕ E125 negatively stained with 1% phosphotungstic acid. Scale bar, 100 nm.

DB110795 with the monoclonal antibody 3D11 and found that it did not produce LPS O antigen (Fig. 2B). These results demonstrate that there is a correlation between the absence of LPS O antigen and resistance to plaque formation by ϕ E125.

A previous study demonstrated that IS407A is active in *B. mallei* during serial subculture in vitro. IS407A integrated into the capsule gene cluster in *B. mallei* DD420 and resulted in a capsule-deficient strain (24). The LPS O-antigen gene clusters of NCTC 120 and DB110795 were analyzed to determine if this 1.2-kb insertion element (IS) was responsible for the lack of LPS O-antigen production by these strains. The nucleotide sequence of the *B. pseudomallei* LPS O-antigen gene cluster is known (23), and it was used to design eight PCR primer pairs that would result in 2-kb amplicons spanning the LPS O-antigen locus in *B. mallei*. Eight 2-kb amplicons were generated when PCR assays were performed with these primer pairs and genomic DNA from *B. pseudomallei* 1026b and *B. mallei* ATCC 23344 (data not shown). When the PCR assays were performed with genomic DNA from NCTC 120 and DB110795, seven 2-kb amplicons and one 3.2-kb amplicon were produced (data not shown). The 3.2-kb amplicons generated using primer pairs 7-1-7-2 (NCTC 120) and 8-1A-8-2A (DB110795) were cloned and sequenced. The sequencing results demonstrate that NCTC 120 and DB110795 harbor IS407A insertions in *wbiE* and *wbiG*, respectively. There was a 4-bp duplication of the sequence 5'-CTGC-3' flanking the insertion site in NCTC 120 and a 4-bp duplication of the sequence 5'-GCAG-3' flanking the insertion site in DB110795.

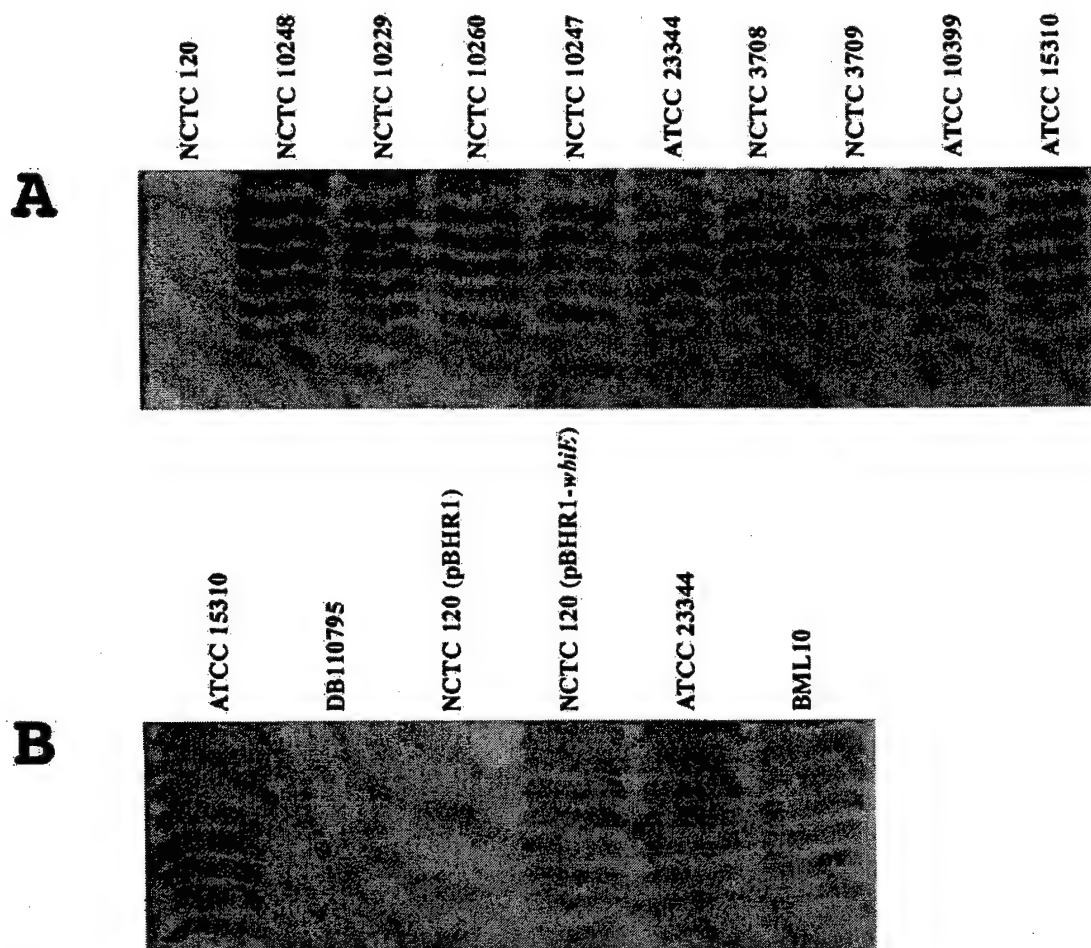


FIG. 2. Immunoblot analysis of *B. mallei* LPS O antigens. Bacteria were washed, resuspended in SDS-PAGE sample buffer, boiled, treated with proteinase K, and subjected to SDS-PAGE. The LPS O antigens were blotted to a polyvinylidene difluoride membrane and reacted with the monoclonal antibody 3D11. (A) LPS O-antigen profiles of NCTC and ATCC *B. mallei* strains. (B) Comparative LPS O-antigen profiles of ϕ E125-resistant and ϕ E125-susceptible *B. mallei* strains. All strains form plaques with bacteriophage ϕ E125 except NCTC 120, DB110795, and NCTC 120 (pBHR1).

Interestingly, the *B. mallei* capsule mutant DD420 harbors an IS407A insertion in *wcbF* that is also flanked by a duplication of the sequence 5'-GCAG-3' (24).

The *wbiE*::IS407A mutation in NCTC 120 was complemented by providing the *wbiE* gene from ATCC 23344 in *trans* on the broad-host-range plasmid pBHR1 (Table 1). Figure 2B shows that NCTC 120 (pBHR1) does not produce LPS O antigen but that NCTC 120 (pBHR1-*wbiE*) does. Furthermore, NCTC 120 (pBHR1-*wbiE*) formed plaques with ϕ E125, but NCTC 120 (pBHR1) did not. These results demonstrate that the lack of LPS O-antigen production by NCTC 120 is due to an IS407A mutation in *wbiE* and that the LPS O antigen is required for plaque formation by ϕ E125.

BML10 is immune to ϕ E125 superinfection and produces LPS O antigen. Lysogenic bacteria are resistant to superinfection by the temperate bacteriophage that they harbor. Following infection, the ϕ E125 genome integrates in the *B. mallei* chromosome at a specific site and becomes a prophage (see below). ATCC 23344 was infected with ϕ E125, and a lysogenic

derivative was isolated and designated BML10. *B. mallei* BML10 spontaneously produced approximately 500 ϕ E125 per ml of broth culture. In comparison, *B. thailandensis* E125 spontaneously produced approximately 1,100 ϕ E125 per ml of broth culture. As shown in Table 2, ϕ E125 does not form plaques on BML10. Whole-cell lysates of ATCC 23344 and BML10 were analyzed by immunoblot analysis with the monoclonal antibody 3D11, and both strains produced a typical LPS O-antigen banding pattern (Fig. 2B). As shown above, NCTC 120 and DB110795 are resistant to infection with ϕ E125 because they do not produce LPS O antigen. BML10, on the other hand, produces LPS O antigen but is still resistant (immune) to ϕ E125 superinfection, probably via a prophage-encoded gene product(s). It should be noted that *B. thailandensis* E125 also harbors the ϕ E125 prophage and is also immune to superinfection with ϕ E125 (Table 2).

Molecular characterization of the bacteriophage ϕ E125 genome. The ϕ E125 genome was digested with *Hind*III, and eight fragments were generated of the following sizes: 1.0, 3.2,

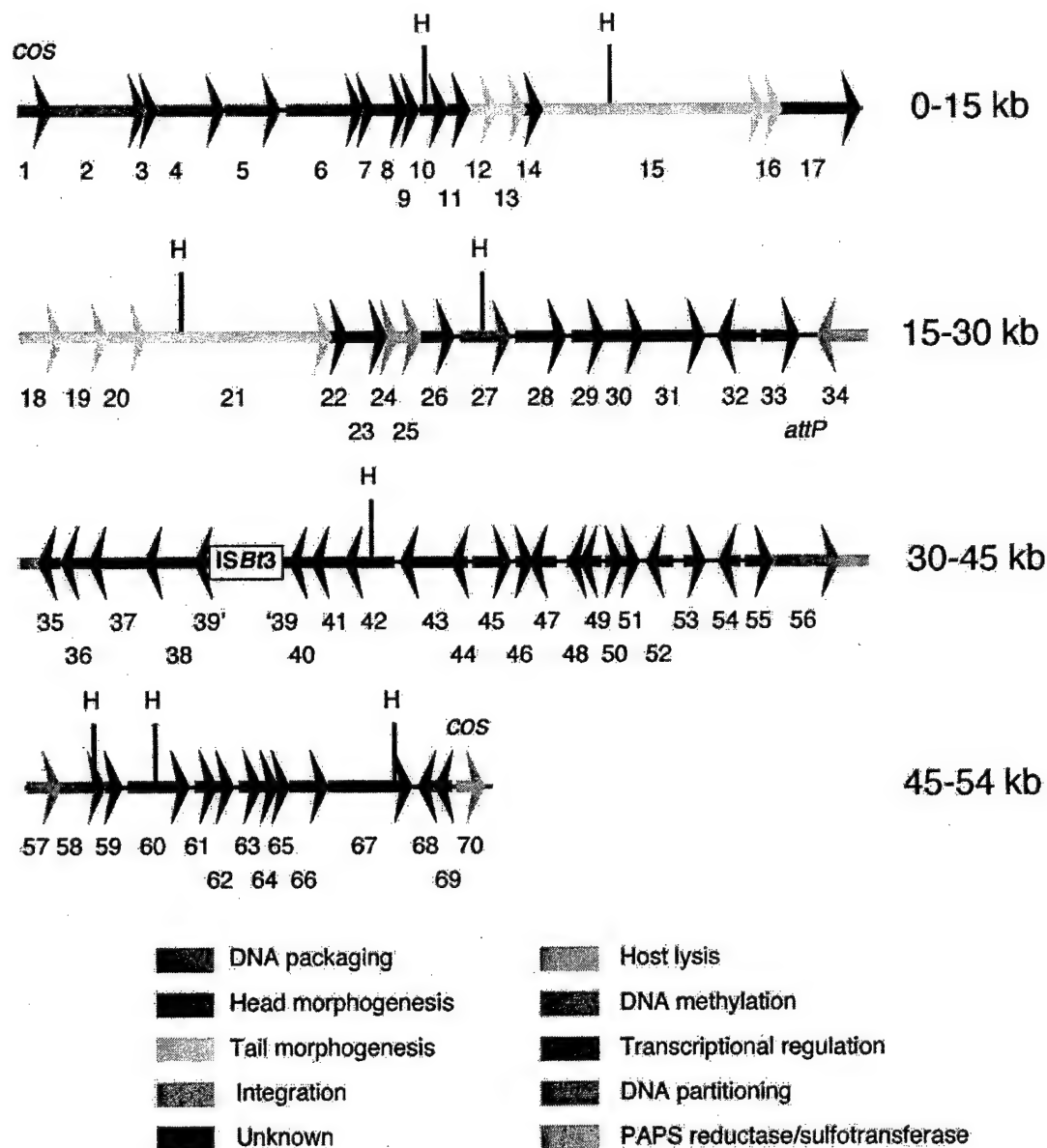


FIG. 3. Physical and genetic map of the bacteriophage ϕ E125 genome. The locations and directions of transcription of genes are represented by arrows, and the gene names are shown below. The locations of *Hind*III endonuclease restriction sites are shown (H), and the insertion sequence *ISBt3* is represented as a rectangle. The locations of the cohesive (*cos*) and bacteriophage attachment (*attP*) sites are shown above and below the ϕ E125 genome, respectively. The putative functions of proteins encoded by ϕ E125 genes are color coded.

4.4, 5.5, 7.3, 9.0, 9.9, and 13.0 kb. The fragments were heated to 80°C, and the 9.0-kb fragment dissociated into two fragments (1.7 and 7.3 kb), suggesting the presence of a cohesive (*cos*) site on this fragment (data not shown). The eight *Hind*III fragments were cloned, and their nucleotide sequences were determined. The nucleotide-sequencing results are depicted schematically in Fig. 3, and pertinent features of ϕ E125 genes and gene products are shown in Table 3.

The ϕ E125 genome is a linear molecule of 53,373 bp in length, and it contains 10-base 3' single-stranded extensions on the left (3'-GCGGGCGAAG-5') and right (5'-CGCCGCTT C-3'), as depicted in Fig. 3. The G + C content of the ϕ E125

genome is 61.2%, which is lower than the 69.3% G + C content of the *B. thailandensis* genome (77). The ϕ E125 genome encodes 70 proteins, and 44% of them show no homology to proteins in the GenBank databases using the BLASTP search algorithm (Table 3 and Fig. 3). The bacteriophage genome also harbors a novel IS3 family insertion sequence (45), designated *ISBt3* (Table 3 and Fig. 3). *ISBt3* is 1,318 bp in length, and it has 27-bp terminal inverted repeats flanked by a 3-bp direct duplication. *ISBt3* integrated into ϕ E125 gene39, suggesting that the encoded protein (gp39) is not essential for a productive lysogenic infection.

Twenty-eight proteins encoded by ϕ E125 are similar to pro-

TABLE 3. Characteristics of bacteriophage ϕ E125 genes and gene products

Gene	Orientation ^a	Start (position)	End (position)	Size of protein (kDa)	Protein function and homologs
1	R	46	531	17.2	Terminase (small subunit); phage GMSE-1 Orf16; 1e-12; AF311659; phage 7201 Orf21; 2e-09; AF145054
2	R	541	2253	64.2	Terminase (large subunit); <i>E. coli</i> YmfN; 1e-162; NP_415667; phage D3 terminase; 1e-125; NP_061498
3	R	2250	2435	6.3	
4	R	2440	3699	46.4	Portal protein; <i>H. influenzae</i> Orf25-like protein; 1e-40; AAF27362; CP-933C Z1849; 1e-40; NP_287334
5	R	3759	4664	31.7	Capsid assembly protein/protease; phage WO Orf7; 8e-44; AB036665; prophage Gifsy-1 STM2605; 2e-34; AE008818
6	R	4767	6074	46.3	Major capsid protein; CP-933N Z1804; 2e-09; NP_287292; CP-933M Z1360; 2e-09; NP_286882
7	R	6134	6319	6.4	
8	R	6326	6892	20.6	
9	R	6892	7218	12.1	Phage HK022 gp9; 3e-08; AF069308
10	R	7211	7633	15.4	Phage HK97 gp10; 4e-20; AF069529 CP-933M Z1368; 1e-19; NP_286890
11	R	7630	7977	12.1	
12	R	8039	8497	16.4	Major tail subunit protein; phage HK97 gp12; 2e-20; NP_037706; <i>E. coli</i> ECs1800; 7e-17; NP_309827
13	R	8519	8989	17.4	Tail assembly chaperone protein; phage HK97 gp13; 9e-06; NP_037708; phage HK97 gp14; 1e-05; NP_037707
14	R	8989	9273	10.0	Phage HK97 gp14; 9e-07; NP_037707
15	R	9287	13351	143.0	Tail length tape measure protein; CP-933P Z6034; 2e-25; NP_287971; <i>E. coli</i> ECs2240; 2e-25; NP_310267
16	R	13348	13686	12.5	Minor tail protein; phage HK97 gp17; 1e-18; NP_037711; phage HK022 gp17; 3e-18; NP_037677
17	R	13695	15083	50.1	
18	R	15080	15763	25.2	Minor tail protein; <i>P. aeruginosa</i> PA0638; 2e-62; G83565; phage N15 gp18; 3e-51; AF064539
19	R	15783	16565	28.6	Tail component protein; <i>P. aeruginosa</i> PA0639; 1e-51; AE004499; phage HK97 gp19; 1e-43; AF069529
20	R	16562	17146	20.1	Tail component protein; <i>Y. pestis</i> YPO2129; 3e-33; AJ414151; phage N15 gp20; 3e-28; AF064539
21	R	17143	20448	118.3	Tail tip fiber protein; <i>Y. pestis</i> YPO2131; < 1e-119; AJ414151; phage N15 gp21; < 1e-119; AF064539
22	R	20445	20759	11.6	
23	R	20759	21493	27.6	
24	R	21536	21748	7.6	Class II holin; phage PS119 gp13; 8e-04; AJ011581; phage PS34 gp13; 8e-04; AJ011580
25	R	21826	22230	14.7	Lysozyme; <i>X. fastidiosa</i> XF0513; 2e-13; AE003900; phage PS119 gp19; 2e-11; AJ011581
26	R	22227	22775	18.6	
27	R	22918	23706	30.2	DNA adenine methylase; phage GMSE-1 Orf10; 1e-40; AF311653; <i>A. lwoffii</i> Alw1 methylase; 5e-19; AF431889
28	R	23815	24693	30.9	
29	R	24875	25435	19.1	<i>P. aeruginosa</i> PA1508; 7e-07; AE004579; <i>Y. pestis</i> YPO0866; 8e-07; AE004579
30	R	25432	26166	26.3	<i>P. aeruginosa</i> PA0822; 5e-23; AE004517; <i>P. aeruginosa</i> PA0823; 4e-09; AE004517
31	R	26194	27285	39.4	<i>P. aeruginosa</i> PA0821; 1e-48; AE004517
32	L	28059	27418	25.0	
33	R	28145	28816	25.3	Plasmid pFKN Orf11; 2e-17; AF359557; plasmid pNL1 Orf520; 3e-04; AF079317
		29062	29014		attP (3' end of tRNA ProUGG)
34	L	30292	29192	42.2	Site-specific integrase; prophage XfP2 XF2530; 6e-09; AE004060; CP-933M Z1323; 1e-08; NP_286846
35	L	30609	30292	11.8	
36	L	31142	30642	19.3	
37	L	32131	31139	37.2	<i>M. tuberculosis</i> Rv2734; 8e-32; NP_217250; <i>N. punctiforme</i> hypothetical protein; 4e-28; AAK68643
38	L	33180	32128	37.9	
39'	L	33314	33195		
tnpB	L	34204	33350	32.6	Transposase (IS3 family); IS868 ORF4; 1e-101; X55075; IS401 transposase subunit; 4e-99; L09108
tnpA	L	34539	34261	10.7	Transposase (IS3 family); IS401 transposase subunit; 6e-29; L09108; IS868 Orf1; 9e-20; X55075
'39	L	34794	34636		

Continued on following page

TABLE 3—Continued

Gene	Orientation ^a	Start (position)	End (position)	Size of protein (kDa)	Protein function and homologs
40	L	35195	34794	14.6	Phage M×8 p77; 0.1; AF396866
41	L	35863	35192	25.1	CP-9330 Z2097; 4e-05; AE005346; CP-933U Z3120; 2e-04; AE005422
42	L	36671	35877	29.8	
43	L	37670	36858	29.7	<i>Y. pestis</i> YPMT1.49c; 1e-38; NC_003134; <i>S. enterica</i> HCM2.0006c; 2e-38; AL513384
44	L	37816	37667	5.5	
45	R	38065	38718	28.6	
46	R	38919	39173	9.1	
47	L	39586	39170	15.5	
48	L	39993	39862	4.7	
49	L	40278	40003	10.4	
50	R	40519	40662	5.0	
51	R	40747	40935	7.0	
52	L	41555	41163	14.4	Repressor protein; <i>X. fastidiosa</i> XF0499; 9e-14; AE003899; prophage e14 protein b1145; 9e-04; F64859
53	R	42006	42338	11.8	
54	L	42821	42567	9.4	
55	R	43007	43447	15.8	Phage phi CTX Orf33; 0.24; BAA36261
56	R	43452	44588	42.1	DNA cytosine methylase; <i>C. freundii</i> Cfr91 methylase; 8e-45; X17022; <i>P. alcaligenes</i> Pac251 methylase; 1e-44; U88088
57	R	44585	45583	36.8	PAPS reductase/sulfotransferase; phage 186 Orf84; 6e-55; U32222; <i>A. pernix</i> APE2075; 8e-07; F72512
58	R	45618	46439	29.9	DNA partitioning protein; <i>R. equi</i> ParA; 1e-06; NP_066815; <i>L. lactis</i> ParA; 2e-05; NP_266252
59	R	46436	46696	9.5	
60	R	46850	47842	36.3	CP-933R Z2397; 7e-06; NP_287824
61	R	48035	48388	13.9	Prophage pi3 protein 45; 4e-05; AL596172; <i>L. monocytogenes</i> lmo2306; 6e-05; CAD00384
62	R	48385	48741	13.2	
63	R	48843	49181	12.6	
64	R	49172	49516	13.1	
65	R	49492	49752	9.8	
66	R	49761	50408	24.1	
67	R	50482	51876	51.3	<i>S. meliloti</i> Sma0594; 6e-11; NP_435557
68	L	52603	52217	14.1	Helix-turn-helix transcriptional regulator; <i>S. meliloti</i> SMc00089; 4e-10; NP_385047; <i>A. tumefaciens</i> AGR_C_1081p; 2e-04; NP_353634
69	L	52857	52600	9.3	<i>P. horikoshii</i> PHS013; 1e-04; NP_142388; <i>P. jensenii</i> Orf10; 0.006; CAC38044
70	R	52916	53272	13.1	Class I holin; <i>X. nematophila</i> prophage Orf7; 1e-16; CAB58450; <i>H. influenzae</i> holin-like protein; 4e-08; AF198256

^a R, right; L, left.

teins encoded by other bacteriophage, prophage, or prophage-like elements (Table 3). Interestingly, there are numerous similarities to HK022 and HK97 (34) and to λ -like cryptic prophages in *E. coli* O157 Sakai (52) and *E. coli* O157 EDL933 (53). Bacteriophage genomes are composed of a mosaic of multigene modules, each of which encodes a group of proteins involved in a common function, such as DNA packaging, head biosynthesis, tail biosynthesis, host lysis, lysogeny, or replication (11, 28, 34, 37). The ϕ E125 genome contains a unique combination of multigene modules involved in DNA packaging, head morphogenesis, tail morphogenesis, and host lysis (Fig. 3 and Table 3). The relative order of these modules in the ϕ E125 genome is similar to that of other *Siphoviridae* genomes (11, 34, 37, 42). Since ϕ E125 possesses both structural and genetic similarities to the λ supergroup group of *Siphoviridae*, it probably should be included with λ , N15, HK97, HK022, and D3 in the λ -like genus (11).

Early bacteriophage gene functions (lysogeny and replica-

tion) are typically located on the right half of *Siphoviridae* genomes, as depicted in Fig. 3 (11). However, the putative lysogeny and replication modules of ϕ E125 appear to be unique relative to other members of the *Siphoviridae*. Some of the unusual proteins encoded by the right half of the ϕ E125 genome include a DNA adenine methylase (gp27), a DNA cytosine methylase (gp56), a 3'-phosphoadenosine-5'-phosphosulfate (PAPS) reductase or PAPS sulfotransferase (gp57), and a chromosome partitioning protein (gp58) (Fig. 3 and Table 3). The ϕ E125 genome also contains two putative holins, gp70 (class I) and gp24 (class II), to coordinate the programmed release of lysozyme (gp25) from the cytoplasm prior to bacteriophage release (72). It is currently unknown if gp70, gp24, or both gp70 and gp24 are required for membrane permeabilization during the ϕ E125 life cycle. Finally, several recently sequenced bacterial genomes also encode proteins with similarities to gp29, gp30, gp31, gp33, gp37, gp43, gp61, gp67, gp68, and gp69 (Table 3), suggesting the presence of prophages or

prophage remnants in these bacterial genomes. Alternatively, ϕ E125 may have acquired these genes via horizontal transfer from a bacterial host, and they may provide a selective advantage to a lysogen harboring this bacteriophage.

ϕ E125 integrates into a proline tRNA (UGG) gene in *B. thailandensis* and *B. mallei*. As with other lambdoid bacteriophages, ϕ E125 DNA probably circularizes at the *cos* sites after it is injected into the bacterial cell and follows one of two possible pathways (14). The circularized genome may replicate and produce bacteriophage progeny (lytic response), or it may integrate into the bacterial chromosome and be maintained in a quiescent state (lysogenic response). Temperate bacteriophage genomes often contain an attachment site (*attP*) that they utilize to integrate into a homologous region on the bacterial genome (*attB*) via site-specific recombination (18). Since ϕ E125 encodes a site-specific integrase (*gp34*), we were interested in identifying where the ϕ E125 genome was integrated in *B. thailandensis* E125 and *B. mallei* BML10 and in determining the nucleotide sequences of *attP* and *attB*.

Chromosomal DNA flanking one side of the ϕ E125 attachment site in *B. thailandensis* E125 was cloned and sequenced (see Materials and Methods). The nucleotide sequence of this region contained a 49-bp sequence that was identical for the ϕ E125 genome and the *B. thailandensis* E125 chromosome. This sequence corresponded to the 3' end of a 77-bp proline tRNA (UGG) gene on the *B. thailandensis* chromosome (Fig. 4A). tRNA genes often serve as target sequences for site-specific integration of temperate bacteriophages, plasmids, and pathogenicity islands (27, 63). Immediately upstream of the proline tRNA (UGG) gene on the *B. thailandensis* chromosome was a divergently transcribed gene designated *orfB* (Fig. 4B). BLASTP results demonstrated that *OrfB* was 52% identical to RSc1539, a probable hydrolase protein from *R. solanacearum*. The *B. thailandensis* proline tRNA (UGG) gene and *orfB* were also present in the *B. mallei* ATCC 23344 genome (<http://www.tigr.org/>), and they were 100 and 91% identical at the nucleotide level, respectively. Downstream of the proline tRNA (UGG) gene in *B. mallei* ATCC 23344 was *orfA*, a gene that encoded a protein with 40% identity to RSc2888, a hypothetical protein from *R. solanacearum* (Fig. 4B). In order to determine if ϕ E125 integrates in the 3' end of the tRNA proline (UGG) gene in *B. mallei*, we designed PCR primers specific for *B. mallei orfA* and ϕ E125 gene34 (Fig. 4B). *B. mallei* ATCC 23344 and ϕ E125 DNA did not yield a PCR product with these primers, but *B. mallei* BML10 did (data not shown). These results, represented schematically in Fig. 4B, demonstrate that bacteriophage ϕ E125 integrates into the 3' end of the proline tRNA (UGG) gene in *B. mallei* and *B. thailandensis*. It should also be noted that attachment at this site leaves the proline tRNA (UGG) gene intact on the right side, as depicted in Fig. 4B.

Survey of *B. thailandensis* strains for the presence of ϕ E125-like prophages. As mentioned above, lysogenic bacteria are immune to superinfection with the same (or similar) bacteriophage that they harbor. The results presented in Table 2 demonstrate that all thirty-two *B. thailandensis* strains in our collection, including E125, are resistant to infection with ϕ E125. To determine if the strains were resistant to infection because they harbored ϕ E125-like prophages, genomic DNA was isolated from all strains and PCR was performed with primer

pairs specific for four distinct regions of the ϕ E125 genome. The primer pairs used were 9.5R and 3.2R (gene9 and gene10), 7.5F and 5.5F (gene21), 18R and 11F (gene42), and 4.4R and 9.5F (gene67). Only ten of the thirty-two *B. thailandensis* strains yielded positive PCR results with these primer pairs (E96, E100, E125, E253, E254, E256, E263, E264, E286, and E293). As expected, E125 was positive for all of the PCR primer pairs. The only other strain that was positive for all four primer pairs was E286. Strains E253 and E264 yielded positive PCR results for two primer pairs, and all of the other strains were positive for three primer pairs. All 10 *B. thailandensis* strains spontaneously produced bacteriophage that formed plaques on *B. mallei* ATCC 23344. Thus, it appears that E96, E100, E253, E254, E256, E263, E264, E286, and E293 all harbor ϕ E125-like prophage and may be immune to superinfection with ϕ E125. On the other hand, 22 *B. thailandensis* strains did not yield a positive PCR product with any of the primer pairs and probably do not harbor a ϕ E125-like prophage. These observations suggest that the molecular mechanism of ϕ E125 resistance in these strains is probably not due to superinfection immunity.

Functional analysis of the putative DNA methyltransferases of ϕ E125. ϕ E125 encodes two proteins, gp27 and gp56, that contain similarities to Type II DNA methyltransferases (Table 3). Site-specific DNA methylation usually leads to the formation of three different products: N6-methyladenine (m6A), 5-methylcytosine (m5C), and N4-methylcytosine (m4C). Some tailed bacteriophage genomes contain unusual or modified DNA bases that may be important in protecting the infecting bacteriophage DNA from host restriction endonucleases (1). gp27 is a putative DNA adenine methylase, and gp56 is a putative DNA cytosine methylase. We were interested in determining if gp27 and gp56 were functional DNA methyltransferases.

The plasmids pCR2.1, pAM1, and pCM1 (Table 1) were transformed into *E. coli* DB24, a strain that is deficient in all of the *E. coli* DNA methylases (36), and DNA methylase dot blot assays were performed with rabbit primary antibodies specific for m6A and m4C. Figure 5A shows that the m6A antibody reacted with genomic DNA samples from λ (positive control) and DB24 (pAM1) but did not react with DB24 (pCR2.1) or DB24 (pCM1). The m6A antibody also reacted with genomic DNA samples from *B. mallei* BML10 and bacteriophage ϕ E125 (Fig. 5A). On the other hand, there was only background reactivity of the m6A antibody with genomic DNA from *B. thailandensis* E125 and *B. mallei* ATCC 23344 (Fig. 5A). It appears that the ϕ E125 m6A methylase has little or no activity in the *B. thailandensis* lysogen but is very active in the *B. mallei* lysogen (Fig. 5A, compare E125 and BML10). It is currently unclear if the *B. mallei* BML10 genome contains m6A or if the positive signal obtained with the m6A antibody is due to the ϕ E125 genome, which also contains m6A (Fig. 5A). Taken together, these results clearly demonstrate that gene27 is expressed in DB24, that gp27 is a functional m6A methylase, and that the ϕ E125 genome contains m6A.

The m4C antibody did not react with genomic DNA from DB24 (pCR2.1), DB24 (pAM1), DB24 (pCM1), or *B. thailandensis* E125, but it did react with DB24 genomic DNA methylated with *M.RsaI* as a positive control (Fig. 5B). This indicates that gene56 is not expressed or is inactive in DB24 (pCM1) and *B. thailandensis* E125. On the other hand, positive

A

5' CGGAGCGTAGCGCAGCCTGGTAGCGCATCTGATT**TGGGAT**
CAGAGGGTCGTAGGTTCTGAATCCTATCGCTCCGACCA 3'

B

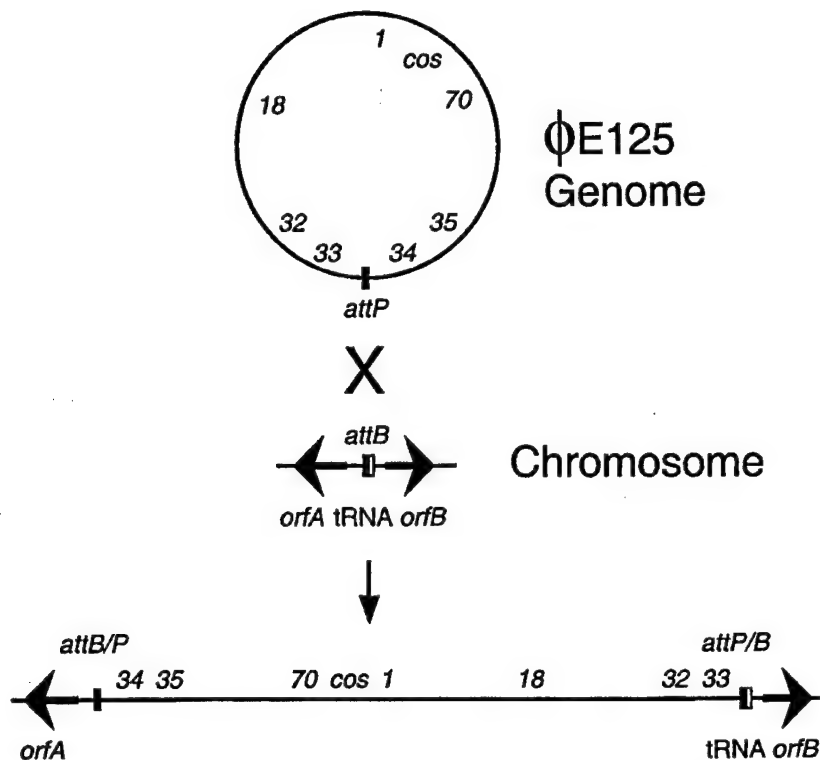


FIG. 4. Bacteriophage ϕ E125 integrates into the proline tRNA (UGG) gene in *B. mallei* and *B. thailandensis*. (A) The nucleotide sequence of the proline tRNA (UGG) gene of *B. mallei* ATCC 23344 and *B. thailandensis* E125. The underlined sequence represents the 49-bp attachment site that is identical in the ϕ E125 genome (*attP*), the *B. mallei* chromosome (*attB*), and the *B. thailandensis* chromosome (*attB*). The location of the anticodon in the proline tRNA gene is shown in bold. (B) Schematic representation of integration of the ϕ E125 genome into the proline tRNA (UGG) gene of *B. mallei* and *B. thailandensis*. The ϕ E125 genome is depicted as a circle, and the approximate locations of gene 1, gene 18, gene 32, gene 33, gene 34, gene 35, and the *cos* site are shown. The *B. mallei* and *B. thailandensis* chromosomes are represented as a line, and the location and direction of transcription of *orfA* and *orfB* are represented by arrows. The 5' end of the proline tRNA (UGG) gene is shown as a thin white rectangle, and the 3' end (the attachment site) is shown as a thin black rectangle. Following site-specific recombination (X), the *orfA* and *orfB* genes are separated by the integrated ϕ E125 prophage.

signals were obtained when the m4C antibody was reacted with genomic DNA from *B. mallei* BML10 and ϕ E125 (Fig. 5B). It is likely that gp56 is an m4C methylase because genomic DNA from *B. mallei* BML10 reacts with the m4C antibody, but *B. mallei* ATCC 23344 genomic DNA does not (Fig. 5B). Alternatively, ϕ E125 infection may activate a cryptic *B. mallei* m4C methylase or a ϕ E125 protein other than gp56 may be responsible for the m4C methylase activity in *B. mallei* BML10. It is not clear if the *B. mallei* BML10 genome contains m4C or if the positive signal obtained with the m4C antibody is strictly

due to m4C methylation of the ϕ E125 genome (Fig. 5B). Further studies will be required to determine the DNA specificities of gp27 and gp56.

DISCUSSION

In this study, we isolated and characterized ϕ E125, a tailed bacteriophage specific for *B. mallei*. The host range of ϕ E125 was examined by using bacteria from three genera of β -*Proteobacteria* (*Burkholderia*, *Pandoraea*, and *Ralstonia*) and five

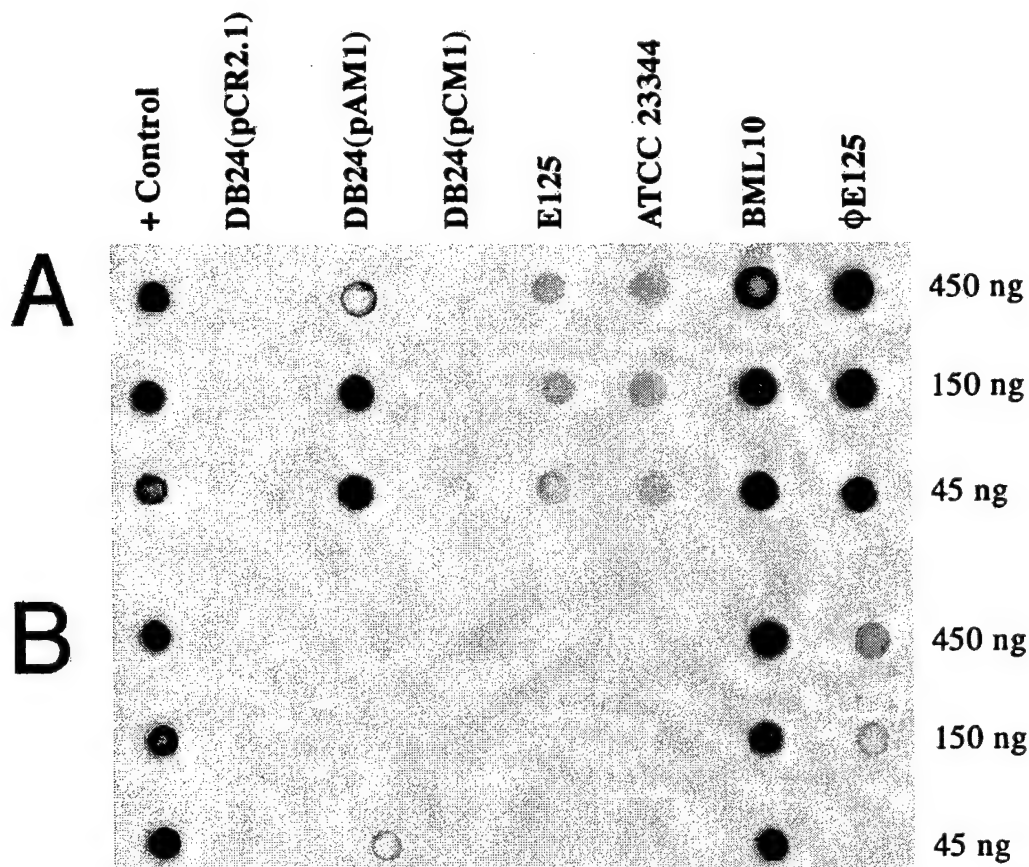


FIG. 5. Dot blot assay to detect genomic DNA methylation using rabbit primary antibodies specific for m6A or m4C. (A) Methylase dot blot assay using polyclonal antibodies specific for m6A. Bacteriophage 2 genomic DNA was used as a positive (+) control. (B) Methylase dot blot assay using polyclonal antibodies specific for m4C. *E. coli* DB24 genomic DNA methylated by *M.RsaI* served as a positive (+) control. The quantities of genomic DNAs spotted on each panel are shown.

genera of γ -Proteobacteria (*Pseudomonas*, *Stenotrophomonas*, *Salmonella*, *Serratia*, and *Escherichia*). In fact, eighteen different *Burkholderia* species were tested, and only *B. mallei* strains were sensitive to ϕ E125 (Table 2). The most-impressive host specificity results were obtained with *B. pseudomallei* and *B. thailandensis*, two species closely related to *B. mallei*. Bacteriophage ϕ E125 did not form plaques on any of the 50 strains of *B. pseudomallei* or 32 strains of *B. thailandensis* tested in this study. Glanders was eradicated from North America in the 1930s and we were able to test only 13 strains of *B. mallei* due to the difficulty of obtaining unique isolates of this species. Nonetheless, the results clearly demonstrate that ϕ E125 specifically forms plaques on *B. mallei*, and we hope to use it, in conjunction with other methods, as a diagnostic tool for *B. mallei*.

The LPS O antigen was required for infection with ϕ E125, suggesting that this molecule is the bacteriophage receptor. This is similar to the λ -like bacteriophage D3, which utilizes the LPS O antigen of *P. aeruginosa* for infection (37, 38). It is surprising that ϕ E125 did not infect *B. pseudomallei* or *B. thailandensis* because the chemical structure of the *B. mallei* LPS O antigen, a heteropolymer of repeating D-glucose and L-talose, is similar to that previously described for these closely

related species (10, 12, 35, 54). In fact, the gene clusters encoding the *B. mallei* and *B. pseudomallei* LPS O antigens are 99% identical at the nucleotide level (12, 23). However, unlike *B. pseudomallei* and *B. thailandensis*, the *B. mallei* LPS O antigen is devoid of an O-acetyl group at the 4' position of the L-talose residue. The chemical structure of the *B. mallei* LPS O antigen is as follows: (3)- β -D-glucopyranose-(1,3)-6-deoxy- α -L-talopyranose-(1-, in which the talose residue contains 2-O-methyl or 2-O-acetyl substituents (12). Our present hypothesis is that *B. pseudomallei* and *B. thailandensis* are resistant to infection with ϕ E125 because the O-acetyl group at the 4' position of the L-talose residue alters the conformation of the LPS O antigen and/or blocks the bacteriophage binding site. *B. pseudomallei* and *B. thailandensis* possess an O-acetyltransferase that is responsible for transferring the O-acetyl group to the 4' position of the L-talose residue. This O-acetyltransferase gene is not present, is not expressed, or is mutated in *B. mallei*. We are currently attempting to identify the *B. pseudomallei* O-acetyltransferase gene and provide it in *trans* to *B. mallei* to see if it O-acetylates the 4' position of L-talose and confers resistance to ϕ E125. Alternatively, inactivation of the O-acetyltransferase gene should make *B. pseudomallei* sensitive to ϕ E125.

It is also possible that *B. pseudomallei* and *B. thailandensis* are immune to superinfection with ϕ E125 because they harbor a ϕ E125-like prophage. The nucleotide sequence of a 1,068-bp *Hind*III fragment from a *B. mallei*-specific bacteriophage produced by *B. pseudomallei* 1026b (ϕ 1026b) was recently obtained and was found to be 98% identical to the 1,068-bp *Hind*III fragment from ϕ E125 (D. DeShazer, unpublished data). However, the nucleotide sequences of other *Hind*III fragments from ϕ 1026b displayed no similarities to ϕ E125, indicating that ϕ 1026b and ϕ E125 are distinct bacteriophages that share regions (modules) of genetic similarity. We found that 10 of the 32 *B. thailandensis* strains in our collection harbor a ϕ E125-like prophage, and the genomic sequence of *B. pseudomallei* K96243 also contains several genes that are nearly identical to ϕ E125 genes (<http://www.sanger.ac.uk/>). Thus, it is clear that some *B. pseudomallei* and *B. thailandensis* strains are lysogenic for a ϕ E125-like bacteriophage and may be immune to superinfection with ϕ E125. It is also important to note that 22 *B. thailandensis* strains in our collection did not possess an ϕ E125-like prophage, suggesting that superinfection immunity alone is not responsible for their resistance to infection with ϕ E125.

In this study, we found that *B. mallei* NCTC 120 and *B. mallei* DB110795 do not produce LPS O antigens due to IS407A insertions in *wbiE* and *wbiG*, respectively. Burtnick et al. (12) have recently obtained identical results with *B. mallei* NCTC 120 and *B. mallei* ATCC 15310, the parental strain of *B. mallei* DB110795. We found that *B. mallei* ATCC 15310 does produce LPS O antigen (Fig. 2A) and does not contain the *wbiG*:IS407A mutation. In fact, the ATCC stock cultures (1964 and 1974) of *B. mallei* ATCC 15310 do not harbor IS407A insertions in *wbiG* (Jason Bannan, personal communication). *B. mallei* DB110795 was obtained by routine laboratory passage of *B. mallei* ATCC 15310 at the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID). The strain used in the study of Burtnick et al. (12) was obtained from USAMRIID and was probably *B. mallei* DB110795, not *B. mallei* ATCC 15310. It was previously reported by members of our group that IS407A integrated into a capsular polysaccharide gene during repeated laboratory passage of *B. mallei* ATCC 23344 (24). Taken together, these results suggest that IS407A transposition may be relatively common during routine laboratory passage of this microorganism. Serial subculture of *B. mallei* on laboratory media results in a loss of virulence for animals (48, 49, 51, 57), and it is tempting to speculate that IS407A transposition is responsible, directly or indirectly, for this phenomenon.

Finally, we found that ϕ E125 genomic DNA contained the methylated bases m6A and m4C (Fig. 5). DNA methylation may protect ϕ E125 DNA from host restriction endonucleases (1), or it may be involved in some other aspect of the ϕ E125 life cycle. We cloned and expressed ϕ E125 gene27 in *E. coli* and found that gp27 was a functional m6A methylase. We were unable to provide direct evidence that gp56 was a m4C methylase, but it was intriguing that ϕ E125 DNA and genomic DNA from a *B. mallei* lysogen contained m4C. It was surprising that genomic DNA from a *B. mallei* lysogen contained m6A and m4C, but genomic DNA from a *B. thailandensis* lysogen did not. We are currently examining the possibility that gp27 and gp56 require host factors for production and/or activity

that are present in *B. mallei* but not in *B. thailandensis*. Type II DNA methylases specifically bind and methylate recognition sequences on a DNA substrate (58). The DNA sequence specificities of gp27 and gp56 are currently unknown, but BLASTP results show that gp56 is similar to cytosine methylases that recognize and methylate the sequence 5'-CCCGGG-3', which occurs nine times in the ϕ E125 genome. ϕ E125 DNA was treated with five restriction endonucleases that recognize this sequence (*Sma*I, *Xma*I, *Cfr*9I, *Psp*AI, and *Xma*CI), and they all cleaved the DNA into nine fragments of the predicted sizes (D. DeShazer and J. A. Jeddeloh, unpublished data). The fact that cleavage was not blocked strongly suggests that this site is not methylated. Further studies are required to determine the specificity of gp27 and gp56 and to understand their role(s) in the ϕ E125 life cycle.

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Burkholderia pseudomallei Class A β -Lactamase Mutations That Confer Selective Resistance against Ceftazidime or Clavulanic Acid Inhibition

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Burkholderia pseudomallei, the causative agent of melioidosis, is inherently resistant to a variety of antibiotics including aminoglycosides, macrolides, polymyxins, and β -lactam antibiotics. Despite resistance to many β -lactams, ceftazidime and β -lactamase inhibitor- β -lactam combinations are commonly used for treatment of melioidosis. Here, we examine the enzyme kinetics of β -lactamase isolated from mutants resistant to ceftazidime and clavulanic acid inhibition and describe specific mutations within conserved motifs of the β -lactamase enzyme which account for these resistance patterns. Sequence analysis of regions flanking the *B. pseudomallei* *penA* gene revealed a putative regulator gene located downstream of *penA*. We have cloned and sequenced the *penA* gene from *B. mallei* and found it to be identical to *penA* from *B. pseudomallei*.

Burkholderia pseudomallei is the causative agent of melioidosis, an endemic disease of Southeast Asia and Northern Australia (6). The severity of the disease can vary from asymptomatic infection to a severe form leading to acute sepsis and death. *B. pseudomallei* is a facultative intracellular pathogen which is able to survive inside phagocytic cells and thereby escape the host's humoral response. The disease can be reactivated after a very long remission (3, 5, 12). Currently, prolonged antibiotic treatment is advised to ensure complete eradication of the organism. Unfortunately, this practice creates a strong positive selection for antibiotic resistant strains resulting in many cases of treatment failure. Many reports have described successful treatment using a combination of β -lactam antibiotics and a β -lactamase inhibitor, such as amoxicillin plus clavulanic acid (19). Livermore, et al. described a clavulanic acid-inhibitable β -lactam resistance phenotype of *B. pseudomallei* (13), and recently, the cloning of *B. pseudomallei* class A and D β -lactamases has been reported (4, 14).

Godfrey et al. described three different phenotypes of clinical isolates from three patients which had undergone antibiotic treatment, and demonstrated that the resistance was due to derepressed β -lactamase production and structural mutations in the enzyme (10). Here, we examine the *B. pseudomallei* *penA* gene encoding a class A β -lactamase in the clinical isolates of *B. pseudomallei* described by Godfrey et al. and from *B. mallei* ATCC 23344. We have identified point mutations in two of the isolates which likely account for their altered phenotypes. Finally, the enzyme kinetics of these mutants were compared to the wild type enzyme.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmid used in this study are shown in Table 1. *B. pseudomallei* strains used in this study were

collected from blood and urine samples from melioidosis patients both before and during antibiotic treatment at Sappasitprasong Hospital, Ubon Ratchatani, Thailand, between 1986 and 1989 and have been described previously (7, 10). All bacterial strains were grown at 37°C on Luria-Bertani (LB) agar or in LB broth. Media used for growing *B. mallei* were supplemented with 4% glycerol. When used, antibiotics were added at the indicated concentrations.

PCR amplification and cloning of PCR products. PCR products were generated in a 100- μ l reaction mixture using the following cycling program: 95°C, 5 min; 95°C 1 min, 55°C 1 min, and 72°C 1 min for 30 cycles; and 72°C 10 min. Mixtures were then held at 4°C. Primers used in the amplification of the 580-bp *penA* from *B. pseudomallei* 1026b were (i) 5'-GCAGCACATCAAGATGATG C-3' and (ii) 5'-GCCGATCGTGTTCATCGTCTA-3'. The primers used in reverse PCR to amplify flanking regions of *penA* using *Xho*I-digested and ligated chromosomal DNA of 1026b were (i) 5'-out (5'-GCATCATCTTGGATGTGCT GC-3') and (ii) 3'-out (5'-GCCGATCGTGTTCATCGTCTA-3'). The primers used to amplify the entire *penA* gene were (i) 5'-*penA* (5'-GAGAGCTGATAC GCTAGCGAG-3') and (ii) 3'-*penA* (5'-GCCGCTTCCGAAGGTTCA-3'). The zeocin resistance gene was amplified with (i) Zeo1 (5'-TGGCCTTTTGCT CACATGTGT-3') and (ii) Zeo2 (5'-TCTAGATCGACCTGCAGGCA-3').

Cloning of β -lactamase genes from *B. pseudomallei* and *B. mallei*. The *penA* gene was amplified from various *B. pseudomallei* mutants and from *B. mallei* using the 5' *penA* and the 3' *penA* primers. The PCR products were subsequently cloned into pCR2.1-TOPO (Invitrogen) as per the manufacturer's instructions. The cloned *penA* genes were transferred from the pCR2.1-TOPO cloning vector to pUCP31T (17) for MIC testing and to pT7Zeo (Invitrogen) for β -lactamase expression. Restriction enzymes and T4 ligase were purchased from BRL/Invitrogen. Plasmids were purified using a QIAprep Spin Miniprep Kit (Qiagen), and chromosomal DNA was prepared using a Wizard DNA purification kit (Promega). When required, PCR products were purified using GenElute PCR DNA purification kit (Sigma).

MIC determination. MICs were determined using agar dilution or E-test strips (AB Biodisk, Solna, Sweden). For agar dilution, Mueller-Hinton agar plates were prepared containing twofold dilutions of antibiotic ranging from 1 to 256 μ g/ml for ampicillin, amoxicillin, and cefazolin; from 0.25 to 128 μ g/ml for amoxicillin plus clavulanic acid (8:1 ratio), cefoxitin, ceftriaxone, and aztreonam; and from 0.25 to 128 μ g/ml for ceftazidime and imipenem. Plates were spotted with approximately 10^4 organisms diluted from overnight liquid cultures and examined after overnight incubation. E-test strips were used as per the manufacturer's instruction.

DNA sequencing and sequence analysis. DNA sequencing was performed by University Core DNA Services (University of Calgary). The CLUSTAL W program (20) was used to align *penA* genes and their translated protein sequences.

Purification of β -lactamase and analysis of enzyme kinetics. The β -lactamase enzyme from *B. pseudomallei* 316a, 316c, and 392f was purified in the following manner. *Escherichia coli* BL21(DE3) (Invitrogen) cells were transformed with p316aT7Z or p392fT7Z, and from the transformants periplasmic proteins were obtained using an osmotic shock procedure.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Strains		
<i>E. coli</i> TOP10	F ⁻ <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80 <i>lacZ</i> Δ M15 Δ <i>lacX74</i> <i>deoR</i> <i>recA1</i> <i>araD139</i> Δ (<i>ara-leu</i>)7697 <i>galU</i> <i>galK</i> <i>rpsL</i> (Str ^r) <i>endA1</i> <i>nupG</i>	Invitrogen
<i>E. coli</i> BL21 (DE3)	F ⁻ <i>ompT</i> <i>hsdS_B</i> (<i>r_B⁻</i> <i>m_B⁻</i>) <i>gal dcm</i> (DE3) pLysS (Cam ^r)	Invitrogen
<i>E. coli</i> BL21 (DE3)pLysS	BL21(DE3)pLysS: F ⁻ <i>ompT</i> <i>hsdS_B</i> (<i>r_B⁻</i> <i>m_B⁻</i>) <i>gal dcm</i> (DE3) pLysS (Cam ^r)	Invitrogen
<i>B. cepacia</i> K56-2	CF isolate, Toronto, Canada	8
<i>B. mallei</i> ATCC 23344	Type strain (human isolate)	USAMRIID ^a
<i>B. pseudomallei</i> 316a	Clinical isolate, wild-type phenotype	8
<i>B. pseudomallei</i> 316c	Clinical isolate, selectively resistant to ceftazidime	8
<i>B. pseudomallei</i> 365c	Clinical isolate, wild-type phenotype	8
<i>B. pseudomallei</i> 365a	Clinical isolate, derepressed phenotype	8
<i>B. pseudomallei</i> 392a	Clinical isolate, wild-type phenotype	8
<i>B. pseudomallei</i> 392f	Clinical isolate, decreased susceptibility to clavulanic acid inhibition	8
<i>B. thailandensis</i> ATCC 700388	Type strain (soil isolate)	16
Plasmids		
pCR2.1-TOPO	Topoisomerase-mediated cloning vector: Ap ^r Km ^r	Invitrogen
pEM7/Zeo	Expression vector, Zeo ^r	Invitrogen
pUCP31T	Broad-host-range vector. OriT pRO1600 ori; Gm ^r	16
p316a31T	pUCP31T containing cloned <i>penA</i> from 316a	This study
p316c31T	pUCP31T containing cloned <i>penA</i> from 316c	This study
p365a31T	pUCP31T containing cloned <i>penA</i> from 365a	This study
p365c31T	pUCP31T containing cloned <i>penA</i> from 365c	This study
p392a31T	pUCP31T containing cloned <i>penA</i> from 392a	This study
p392f31T	pUCP31T containing cloned <i>penA</i> from 392f	This study
pJES307	pT7-7 derivative, expression vector with T7 promoter	15
pT7Zeo	pJES307 derivative with disrupted <i>bla</i> _{TEM-1} , Zeo ^r	This study
p316aT7Z	pT7Zeo containing cloned <i>penA</i> from 316a	This study
p316cT7Z	pT7Zeo containing cloned <i>penA</i> from 316c	This study
p392fT7Z	pT7Zeo containing cloned <i>penA</i> from 392f	This study

^a U.S. Army Medical Research Institute of Infectious Diseases

For *B. pseudomallei* 316c β -lactamase purification, *E. coli* BL21(DE3)LysS (Invitrogen) was used in an effort to obtain higher β -lactamase expression. For osmotic shock, four liters of each *E. coli* transformant was grown overnight, and cells were harvested by centrifugation and resuspended in 30 to 50 ml of 0.5 M sucrose for approximately 15 min. Periplasmic proteins were released by gently resuspending centrifuged cells in 20 ml of sterile distilled water. The periplasmic protein extracts were filter-sterilized and adjusted to 40 mM Tris-HCl, pH 8.5, to a final volume of 10 ml. The adjusted extracts were loaded into a Q-Sepharose fast flow 16/20 chromatography column. The *B. pseudomallei* β -lactamase enzyme was collected in the pass-through fraction. The pass through fraction was then concentrated to 2 ml with a Centrprep Centricon-10 and loaded into a MonoS HR 5/5 fast-performance liquid chromatography column (Amersham Pharmacia). The β -lactamase fraction was then eluted with a 0 to 2 M NaCl gradient to obtain a pure fraction of the enzyme. The pH of the enzyme extract was adjusted to 7.0, and this material was used for kinetic studies.

Enzyme purity was assessed by SDS-14% PAGE (data not shown). The kinetic analysis of β -lactam hydrolysis was performed with a Beckman DU640 spectrophotometer using 0.1 M phosphate buffer, pH 7.0.

Competition assays were performed in a total volume of 500 μ l of buffer in 5- or 10-mm path length quartz cuvettes. Reporter (nitrocefin) was added to a final concentration of 100 μ M and inhibitor to a concentration of 50 or 100 μ M. The extinction coefficients ($\Delta\epsilon$) and UV absorption wavelength of each antibiotic used in this study were as follows: nitrocefin, +15,000 M⁻¹ cm⁻¹ and 482 nm; ampicillin -1,100 M⁻¹ cm⁻¹ and 232 nm; amoxicillin, -1,100 M⁻¹ cm⁻¹ and 232 nm; cefazolin, -7,900 M⁻¹ cm⁻¹ and 260 nm; ceftiofur, -7,700 M⁻¹ cm⁻¹ and 260 nm; ceftriaxone, -9,400 M⁻¹ cm⁻¹ and 260 nm; ceftazidime, -8,660 M⁻¹ cm⁻¹ and 260 nm; aztreonam, -640 M⁻¹ cm⁻¹ and 318 nm; and imipenem, -9000 M⁻¹ cm⁻¹ and 300 nm.

K_m and V_{max} were calculated using nonlinear regression analysis by Prism software. The k_{cat} was obtained using the known amount of enzyme measured by bicinchoninic acid protein assay (Pierce, Rockford, Ill.). K_i was obtained using the method described by Galleni et al. (9) and was used as K_m when the hydrolysis rate could not be measured.

Nucleotide sequence accession numbers. The *penA* sequences were submitted to GenBank under accession numbers AY032868, AY032869, AY032870, AY032871, AY032872, AY032873, and AY032874.

RESULTS

Reverse PCR and flanking region of the *penA* gene. Reverse PCR was performed in order to obtain the sequence of flanking regions of the *penA* gene. The orientation of *penA* and flanking genes are shown in Fig. 1. The *penA* gene is downstream of the *nlpD* gene which is presumably involved in lipoprotein synthesis and is upstream of a putative regulator gene, *penR*. The *nlpD*, *penA*, and *penR* genes have the same orientation. The intergenic region between *nlpD* and *penA* and that between *penA* and *penR* are approximately 150 and about 700 bp, respectively.

DNA sequence analysis. The PCR amplified *penA* gene from six different isolates of *B. pseudomallei* and *B. mallei* ATCC 23344 was sequenced and compared using the CLUSTAL W program (Fig. 2). The DNA sequences of these seven strains



FIG. 1. Orientation of *penA* and *penR* in *B. pseudomallei* and *B. mallei*. *nlpD*, putative enzyme involved in lipoprotein synthesis; *penA*, class A β -lactamase gene; repeats, inverted repeats (not to scale); *penR*, putative regulator gene.

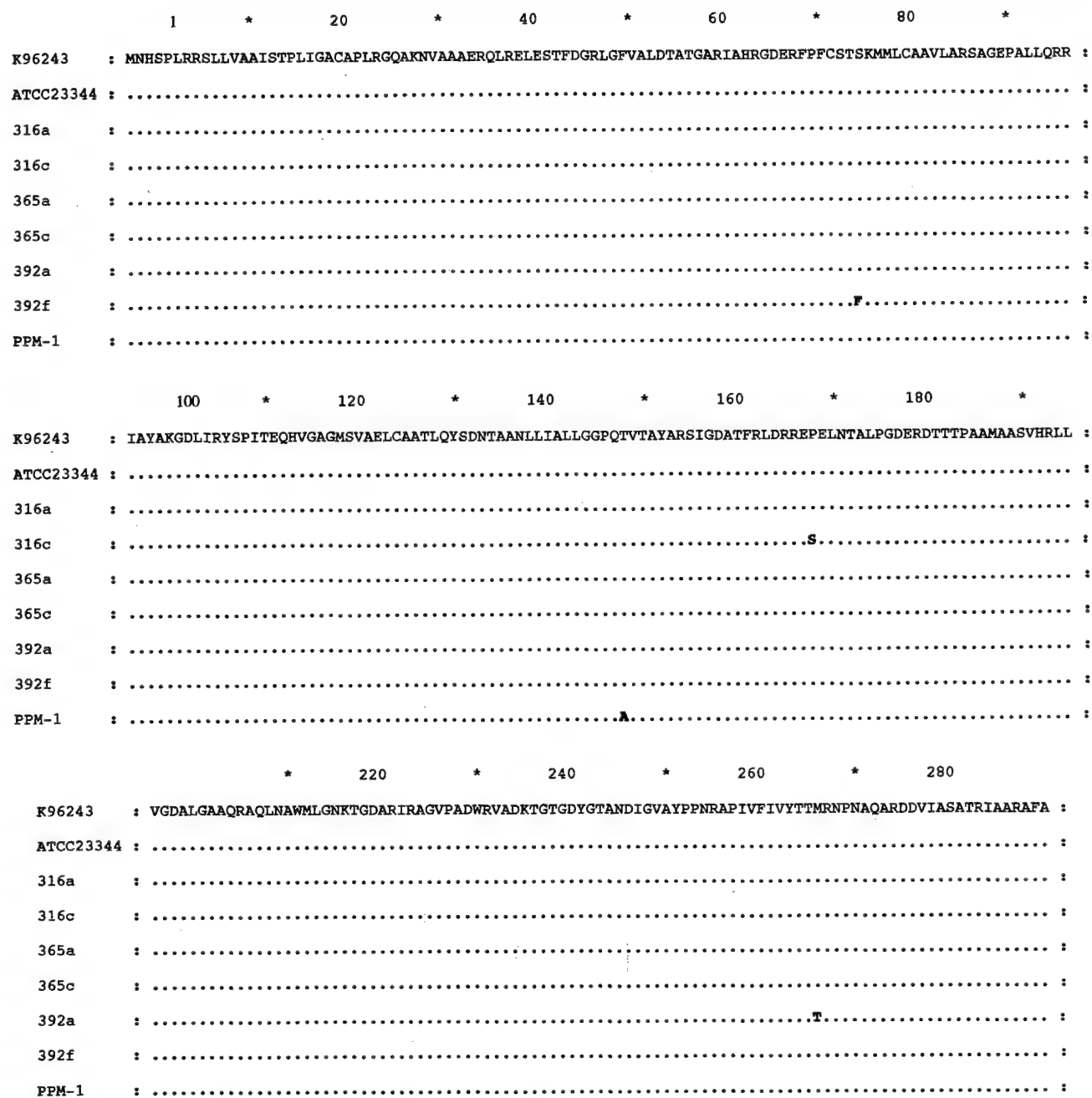


FIG. 2. CLUSTAL W alignment of nine β -lactamases from *B. pseudomallei* and *B. mallei*. K96243, *B. pseudomallei* K96243; ATCC 23344, *B. mallei* ATCC 23344; PPM-1, *B. pseudomallei* Hong Kong strain (4). Dots indicate amino acids identical to those in the sequence for strain K96243. Letters indicate changed amino acids. Amino acids are numbered in accordance with the Ambler (ABL) numbering scheme (Ambler et al., letter).

were almost identical in that only a few single base changes were identified. The presumptive translated protein sequences were identical between *B. mallei* and *B. pseudomallei* strains 316a (wild-type phenotype), 365a (derepressed), and 365c (wild-type phenotype). When strain 316c (ceftazidine resistant) was compared to strain 316a (wild-type phenotype), a single nucleotide change (C to T) was found resulting in a change of proline to serine at position 167 (P167S, ABL numbering scheme [R. P. Ambler, A. F. Coulson, J. M. Frere, J. M.

Ghuysen, B. Joris, M. Forsman, R. C. Levesque, G. Tiraby, and S. G. Waley, Letter, Biochem. J. 276:269-270, 1991]). The clavulanic acid resistant strain 392f had a single nucleotide change (C to T) at S72F. Nucleotide changes in both 316c and 392f resulted in amino acid changes within conserved regions of class A β -lactamases. A single base change (T to C) in strain 392a (wild-type phenotype) resulted in a substitution of methionine with threonine (M266T) and was outside of the conserved regions of class A β -lactamases. Sequence comparison

TABLE 2. MICs ($\mu\text{g/ml}$) of different β -lactams for *E. coli*, *B. pseudomallei*, and *B. mallei*

Strain	MIC ^a ($\mu\text{g/ml}$)								
	AMP	AMX	AMC	CFZ	CRO	FOX	CAZ	ATM	IPM
TOP10	4	4	4	2	<1	4	0.25	<1	0.25
TOP10(p316a31T)	>256	>256	128	256	4	4	2	8	0.25
TOP10(p316c31T)	256	>256	>128	>256	4	>128	4	2	1
TOP10(p365a31T)	>256	>256	32	256	16	8	4	8	0.25
TOP10(p365c31T)	256	>256	32	128	16	8	4	8	0.25
TOP10(p392a31T)	128	>256	8	128	4	8	2	8	0.25
TOP10(p392f31T)	128	>256	16	64	4	8	1	8	0.25
ATCC 23344	64	128	8	>256	8	>128	1	32	0.25
316a	64	128	8	>256	8	>128	4	32	0.5
316c	64	128	8	>256	8	>128	64	32	0.5
365a	>256	>256	128	>256	128	>128	16	>256	2
365c	64	128	128	>256	128	>128	16	>256	0.5
392a	64	128	16	>256	4	>128	2	32	0.5
392f	64	128	32	>256	8	>128	4	64	0.5

^a Abbreviations for antibiotics: AMP, ampicillin; AMX, amoxicillin; AMC, amoxicillin-clavulanic acid; CFZ, cefazolin; CRO, ceftriaxone; FOX, cefoxitin; CAZ, ceftazidime; ATM, aztreonam; IPM, imipenem.

of the putative regulator region in all of the strains examined did not reveal any differences at the amino acid level (data not shown).

MIC determination in parental strains and their corresponding clones. The MICs of ten different β -lactam antibiotics were determined and compared in pairs of *B. pseudomallei* and *E. coli* TOP10 containing the corresponding *penA* clone. The results are shown in Table 2. The MICs of ceftazidime for both 316c and TOP10(p316c31T) were relatively high, although that for TOP10(p316c31T) was not significantly higher than many of the other *E. coli* TOP10 *penA* clones. Also, both 392f and TOP10(p392f31T) showed a small but consistent decrease in susceptibility to clavulanic acid inhibition compared with 392a or TOP10(p392a31T).

β -Lactamase purification and kinetic parameters. Enzyme obtained from periplasmic extracts and subjected to ion exchange column purification yielded β -lactamase with greater than 90% purity. The enzyme preparations were used to examine the kinetics of β -lactam hydrolysis in 3 of the *B. pseudomallei* strains. In general, the β -lactams used in this study could be divided into five groups. The first group consisted of "good" substrates, such as nitrocefin, cefazolin and ceftriaxone, which exhibited high k_{cat}/K_m . The second group represented poor substrates, such as ampicillin and aztreonam. The third group were very poor substrates, such as amoxicillin, in that the hydrolysis rate could not be measured but could be derived by using the competitive hydrolysis method. The fourth group consisted of nonsubstrates as the enzyme could not recognize those β -lactams either as substrates or inhibitors. The last group was the inhibitor group and consisted of a single substrate, clavulanic acid. The hydrolysis rates of nine different β -lactams, representing the five groups described above, were examined using the "good" substrate nitrocefin as a reporter and the K_i obtained from these competitive analysis experiments was used as a K_m for comparison. The kinetic parameters obtained from β -lactamases from three of the *B. pseudomallei* strains are shown in Table 3. Ceftazidime was not recognizable by 316a and 392f enzymes; however, it was a substrate for the 316c enzyme, in that it was recognizable via competitive hydrolysis, albeit very poorly. Ampicillin and az-

treonam were very poor substrates for the 316c enzyme yet good substrates for 316a and 392f. The K_m (K_i) of 392f for clavulanic acid was about fivefold higher than that of 316a indicating lower affinity of the 392f enzyme for clavulanic acid; however, the 316c enzyme had the highest K_m for clavulanic acid among the three strains.

DISCUSSION

This study examines the *penA* gene and the class A β -lactamase enzyme which it encodes from several *B. pseudomallei* clinical isolates. In addition, we have sequenced the *penA* gene from *B. mallei* and have found it to be identical to that found in *B. pseudomallei*. We have shown that the β -lactamase resistant phenotype in *B. pseudomallei* can be attributed to amino acid changes in conserved regions of the β -lactamase enzyme.

Although the sequence of *B. pseudomallei penA* has recently been reported (4), reverse PCR experiments revealed a unique arrangement of the *penA* structural gene with a putative regulator downstream and in the same orientation. Sequence analysis of the putative regulator region did not reveal any differences between all *B. pseudomallei* strains examined at the amino acid level suggesting that the observed derepressed phenotypes are not a result of mutations within this region and that other factors contribute to the elevated enzyme levels in these strains.

The approximately 700-bp region which separates the *penA* gene and the putative regulator contains repeats and inverted repeats and may possibly contain unknown regulatory features. This region remains a target for further studies aimed at understanding the regulation of the β -lactamase enzyme.

The *penA* gene in *Burkholderia spp.* encodes a class A β -lactamase which is susceptible to clavulanic acid inhibition. The predicted protein sequence contains all four conserved motifs found in other class A enzymes—namely, SXXK, SDN, omega loop (EXXLN), and KTG motifs (11)—and according to its activity, *penA* would be classified in the Bush group 2e (2, 4). The enzymes from two strains, 316c and 392f, had mutations that resulted in amino acid changes within the conserved motifs of the catalytic site. The mutation of 316c at the omega

TABLE 3. Kinetic parameters of *B. pseudomallei* β -lactamases^a

Substrate	PenA	V_{max}	K_m	k_{cat}	k_{cat}/K_m
Nitrocefin	316a	159 \pm 7.4	10.9 \pm 2.1	4.91	446
	316c	67.6 \pm 4.2	3.34 \pm 0.7	1.01	303
	392f	351 \pm 7.2	38 \pm 2.1	46.2	1,214
AMP	316a	1,316 \pm 114	126 \pm 29.5	0.18	1.45
	316c	NM	13.8	0.04	2.74*
	392f	4,087 \pm 601	1,051 \pm 237	37.2	35.4
AMX	316a	NM	2.22*	NM	NM
	316c	NM	0.88*	0.09	96.9*
	392f	NM	74.6*	NM	NM
CFZ	316a	1,024 \pm 106	59.5 \pm 17.8	21.35	359
	316c	168 \pm 5.9	12.6 \pm 1.8	0.63	50
	392f	2,390 \pm 383	255 \pm 52.8	78.6	307
FOX	316a	NH	NR	NH	NH
	316c	NM	15.4*	0.02	1.02*
	392f	NH	NR	NH	NH
CRO	316a	1,514 \pm 389	287 \pm 117	63.1	219
	316c	253 \pm 29.4	29.4 \pm 8.5	0.95	32.4
	392f	802 \pm 128	138 \pm 34.4	26.3	191
CAZ	316a	NM	NR	NM	NM
	316c	NM	10.3*	0.02*	2.33*
	392f	NM	NR	NM	NM
ATM	316a	1,645 \pm 211	179 \pm 61.4	3.2	17.9
	316c	NM	48.1*	0.15*	3.2*
	392f	1,939 \pm 144	336 \pm 51.9	17.6	52.5
IPM	316a	NH	NR	NH	NH
	316c	NH	NM**	NH	NM**
	392f	NH	NR	NH	NH
CLA	316a	ND	0.61*	ND	ND
	316c	ND	17.7*	ND	ND
	392f	ND	3.18*	ND	ND

^a Units are μMs^{-1} for V_{max} , μM for K_m , s^{-1} for k_{cat} , and $\text{mM}^{-1}\text{s}^{-1}$ for k_{cat}/K_m . CLA; clavulanic acid. Other antibiotic abbreviations are as listed in Table 1. NR, antibiotic was not recognized by that particular enzyme; NM, the rate of hydrolysis is too slow to be measured accurately; ND, not determined; NH, hydrolysis was not detected after 30 minutes. *, K_i was used as K_m ; **, the enzyme was completely inactivated by imipenem and no nitrocefin hydrolysis was detected.

loop (P167S) may explain the observed ceftazidime resistance as this mutation has been shown to be associated with ceftazidime resistance in *K. pneumoniae* (15). Although the rates of ceftazidime hydrolysis by β -lactamase from 316c and 316a were not directly measurable, the 316c enzyme could recognize ceftazidime as competitive substrate and thereby allowed calculation of K_i , k_{cat} , and k_{cat}/K_m ratio values. It is likely that the increased affinity for ceftazidime of the 316c enzyme may account for the increased resistance of this strain to this antibiotic.

The 392f enzyme also contained a point mutation in a conserved motif resulting in a S72F mutation. Although the phenylalanine at this position can be found in many β -lactamases, it is convincing that in this case, the change resulted in a decreased susceptibility to clavulanic acid inhibition in both *B. pseudomallei* strain 392f and in *E. coli* TOP10(p392f31T) as the K_i for clavulanic acid was higher for the 392f enzyme than the wild type, 316a enzyme. The higher K_i would indicate decreased affinity of clavulanic acid, resulting in a higher MIC for amoxicillin plus clavulanic

acid for strain 392f and *E. coli* TOP10(p392f31T) compared to strain 392a and TOP10 (p392a31T).

The K_i obtained for clavulanic acid and strain 316c was higher than for strains 392f and 316a, which may explain decreased susceptibility to clavulanic acid inhibition. However, the MIC of amoxicillin/clavulanic acid in 316c was not higher than 392f. This may be explained by the fact that 316c enzyme hydrolyzed amoxicillin more poorly than the wild type enzyme, so the decreased susceptibility to clavulanic acid inhibition could not raise the MIC of amoxicillin/clavulanic acid.

The MICs for *B. pseudomallei* *penA* genes cloned into *E. coli* TOP10 may not accurately reflect actual enzyme activity in *B. pseudomallei*. Efflux mechanisms and/or differences in outer membrane permeability may alter periplasmic β -lactam concentrations and thus may affect apparent enzyme activity.

While this work was being reviewed the cloning of a class D β -lactamase from *B. pseudomallei* was reported (14). The authors reported increased transcription of the class D β -lactamase gene in laboratory generated ceftazidime resistant mu-

tants. However, extracts of *E. coli* carrying the cloned gene from parent and mutant showed no detectable ceftazidime or imipenem hydrolyzing activity. Thus, the role of the *B. pseudomallei* class D β -lactamase in β -lactam resistance remains unclear.

Other factors outside the coding region of the *penA* gene may also contribute to highly resistant phenotypes as seen in 365a and 365c. Currently, we are studying the function of *penR*, the putative regulator and the intergenic 700bp region on the expression of β -lactamase in *B. pseudomallei*.

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Identification of Genes Encoding Secreted Proteins Using Mini-*OphoA* Mutagenesis

Mary N. Burtnick, Paul J. Brett, and Donald E. Woods

1. Introduction

Protein fusions are invaluable tools for the genetic studies involving the mechanisms of protein export in bacteria. In 1985, Hoffman and Wright developed an in vitro fusion approach that allowed for fusions of the gene encoding *Escherichia coli* alkaline phosphatase to a variety of cloned genes (1). The modified *phoA* gene employed in these studies, designated '*phoA*', resulted in the production of a highly active alkaline phosphatase protein missing its signal sequence (1). This approach is based on the fact that bacterial alkaline phosphatase is normally periplasmic and must be located extracytoplasmically to be active, i.e., export is essential for high levels of alkaline phosphatase activity (1). Through the fusion of '*phoA*' to portions of heterologous genes containing signal sequences, export from the cytoplasm and subsequent PhoA activity can be observed (1).

The utility of the *phoA* fusion approach was extended with the construction of *TnphoA* (7733 bp), a Tn5 based transposon with a truncated *phoA* gene at one end (2). *TnphoA* randomly generates '*phoA*' fusions upon integration into a recipient bacterial chromosome (2,3). Isolation of mutants harboring active PhoA fusions can be identified easily as they appear blue on agar plates containing the chromogenic substrate 5-bromo-4-chloro-3-indolyl phosphate (XP). Such gene fusions result in the expression of hybrid proteins with PhoA activity if the gene forming the fusion encodes an extracytoplasmic product, i.e., a membrane, periplasmic, outer membrane, or secreted protein.

In 1990, De Lorenzo et al. constructed mini-Tn5*phoA*, a mini-Tn5 derivative with the '*phoA*' gene from *TnphoA* (4). Mini-Tn5*phoA* possesses a transposase (*tnp**) external to the mobile element and is about half the size of

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Tn ϕ oA (4). These features simplify genetic analysis by ensuring stability of the mini-Tn5 ϕ oA integration in the recipient chromosome and increasing the ease of cloning. More recently, a broad host range, self cloning plasmid containing the ϕ oA gene has been constructed (5). The ϕ oA gene from Tn ϕ oA (3) was PCR amplified and ligated it into the plasmid pTnmodOGm (6), resulting in a construct designated mini-O ϕ oA (see Fig. 2) (5). Similar to the mini-Tn5 derivatives, plasmids include the presence of a cognate transposase outside of the inverted repeats allowing for integration into the recipient chromosome without the transposase thereby avoiding additional genetic rearrangements (6). In addition, plasmids possess a pMB1 conditional origin of replication and multiple cloning sites within its inverted repeats that allow for the rapid cloning of DNA flanking the integration site (6). The mini-O ϕ oA fusion system works in the same manner as Tn ϕ oA and mini-Tn5 ϕ oA, however, the presence of an origin of replication that allows for self cloning of the DNA flanking mini-O ϕ oA confers a significant advantage (5). This system simplifies and expedites the identification, cloning and sequence analysis of genes encoding extracytoplasmic products. Figure 1 shows the steps in formation of an active gene fusion using a self-cloning mini-transposon carrying the ϕ oA gene. Figure 2 schematically represents the pmini-O ϕ oA plasmid.

We have optimized the mini-O ϕ oA system for use in phosphatase-negative strains of three *Burkholderia* spp. (5,7), and this system should prove useful in most gram negative bacteria. Described here are the materials and methods used for the identification and characterization of genes encoding extracytoplasmic products using mini-O ϕ oA. See Fig. 3 for an overview of this approach.

2. Materials

Unless otherwise stated chemicals were purchased from Sigma-Aldrich Canada (Oakville, ON, Canada). Tryptone and yeast extract were purchased from Difco (Detroit, MI, USA).

2.1. Conjugation and Screening

1. XP stock solution: 40 mg/mL in deionized water, filter sterilize through a 0.22 μ m filter (Millipore Corp., Mississauga, ON, Canada). Light sensitive: wrap in tin foil and store at -20°C.
2. Gentamicin (Gm) stock solution: 20 mg/mL in deionized water, filter sterilize through 0.22 μ m filter (see Note 1). Store at -20°C.
3. Streptomycin (Sm) stock solution: 100 mg/mL in deionized water, filter sterilize through 0.22 μ m filter (see Note 2). Store at -20°C.
4. Luria-Bertani (LB) Broth: 10 g tryptone, 5 g yeast extract and 5 g sodium chloride (NaCl), dilute to 1 L with deionized water (see Note 3). Store at room temperature.

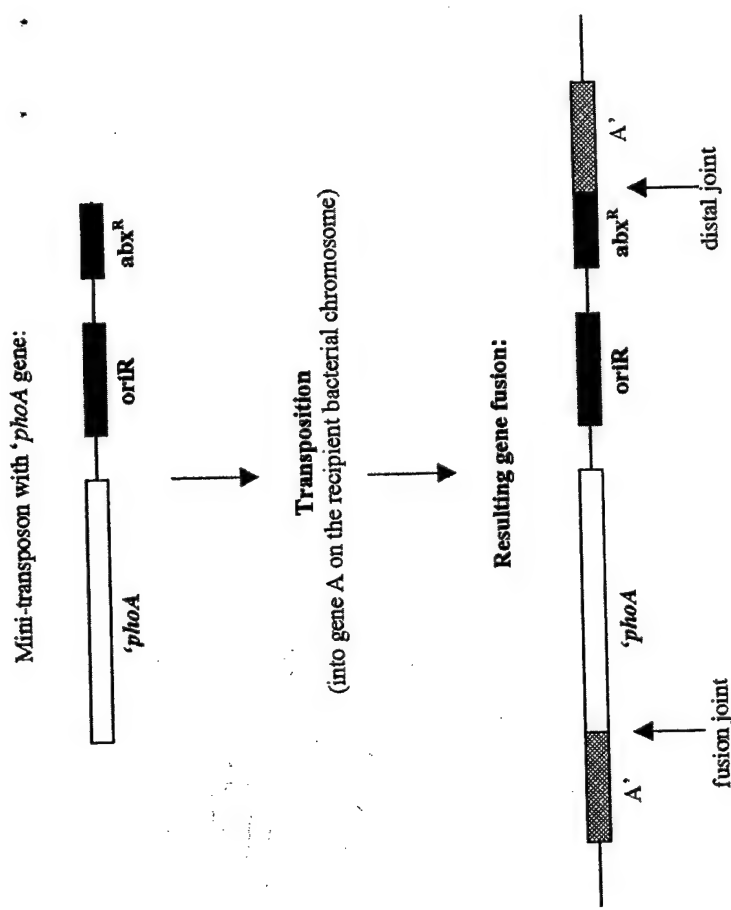


Fig. 1. Formation of an active *phoA* gene fusion using a self-cloning mini-transposon carrying a truncated *phoA* gene. *phoA*: modified *E. coli* alkaline phosphatase gene minus the signal sequence; *oriR*: origin of replication that allows for self-cloning; *abx^R*: antibiotic resistance cassette appropriate for selection of transposition events in the recipient bacterial species in question. An active insertion into gene A results in interruption of the gene and the production of a hybrid protein from A-*phoA* fusion.

5. LB agar: LB broth plus 1.5% agar (15 g/L). Autoclave. Cool agar before pouring plates. Store at 4°C.
6. LBGmXP agar: Prepare low salt LB agar, cool to 55°C. Add Gm to a final concentration of 20 μ g/mL, and XP to a final concentration of 40 μ g/mL. Wrap in tin foil and store at 4°C.
7. Fresh LBGm agar plate of the donor bacterial strain, SM10 (pmini-O ϕ oA).
8. Fresh LBSm agar plate of the recipient bacterial strain.
9. LBSmGmXP agar: Prepare low salt LB agar, cool to 55°C. Add Sm to a final concentration of 100 μ g/mL, gentamicin to a final concentration of 20 μ g/mL, and XP to a final concentration of 40 μ g/mL. Wrap in tin foil and store at 4°C.
10. 40% Glycerol. Autoclave to sterilize. Store at room temperature.

Conjugation of SM10 λ pir (pmini-*OphoA*) with recipient bacterial strain.

Selection for PhoA+ transconjugates.

Cloning of DNA flanking mini-*OphoA* integrations.

Sequence analysis.

Fig. 3. A summary of the steps in mini-*OphoA* mutagenesis and analysis of transconjugates with PhoA activity (PhoA+).

1. LBSmGmXP agar (see Subheading 2.1., item 9).
2. LBGm and LBSm: LB broth with gentamicin 20 μ g/mL or streptomycin 100 μ g/mL, respectively.
3. 15-mL Polypropylene round bottom "snap-cap" tubes (Starstaedt).
4. Wooden toothpicks. Autoclave to sterilize.
5. Genomic DNA isolation protocol of your choice. We use Wizard™ Genomic DNA Isolation kit (Promega, Madison, WI, USA).
6. Restriction endonucleases. See Fig. 2, mini-*OphoA* map for positions restriction endonuclease cleavage sites.
7. 3 M Sodium acetate (NaOAc) pH 4.6. Autoclave. Store at room temperature.
8. 100% Absolute ethanol, store at -20°C and 70% ethanol, store at room temperature.
9. Sterile deionized water.
10. T4 DNA Ligase and 5X Ligase Buffer.
11. Chemically competent or electrocompetent *E. coli* cells. We use Top10 *E. coli* (Invitrogen) or electrocompetent High Efficiency *E. coli* DH5 α (GibcoBRL).
12. LBGmXP agar (see Subheading 2.1., item 6).
13. Plasmid DNA isolation protocol of your choice. We use the QIAprep plasmid miniprep kit (QIAGEN, Mississauga, ON, Canada).
14. 0.8% Agarose gel, appropriate buffers and gel running apparatus. See Sambrook et al. for standard procedures (8).
15. Sequencing primers (5):
 - a. Pho-LT 5'-CAGTAATATCGCCCTGAGCAGC-3'
 - b. Gm-RT 5'-GCCGCGGCCAATTCGAGCTC-3'
16. DNA sequence analysis software and BLASTX program (9): www.ncbi.nlm.nih.gov/blast/index.html.

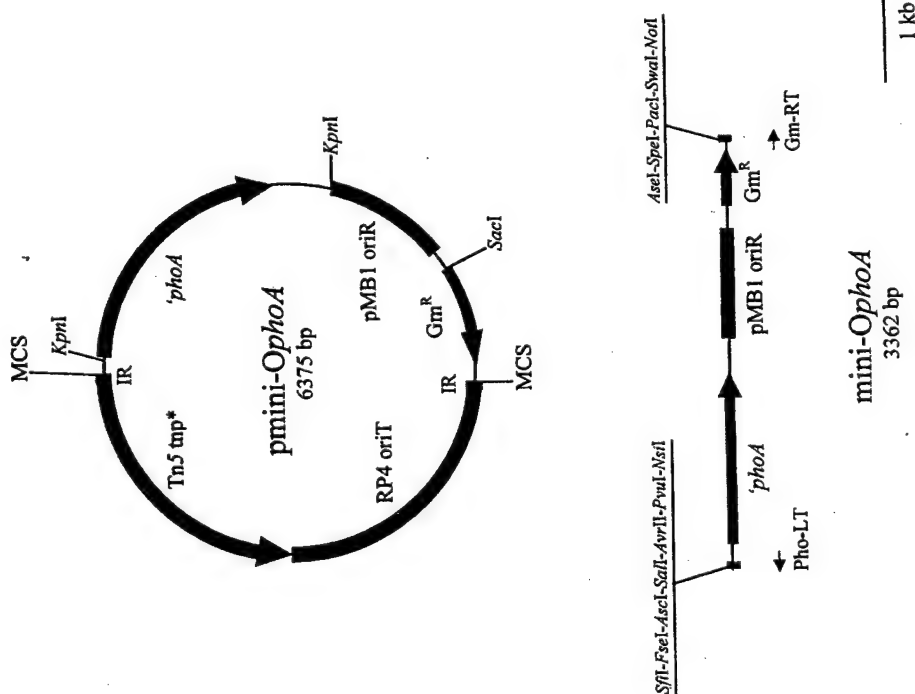


Fig. 2. (A) Schematic of the pmini-*OphoA* plasmid. *phoA*, *E. coli* alkaline phosphatase gene lacking the signal sequence; pMB1 oriR, origin of replication; Gm^R, gentamicin resistance gene; RP4 oriT, origin of transfer; Tn5 tnp*, Tn5 transposase; IR, Tn5 inverted repeats; MCS, multiple cloning sites. (B) mini-*OphoA*, the portion of the plasmid that integrates into the chromosomal DNA of the bacterial recipient. Restriction endonuclease cleavage sites of the MCSs are shown. Pho-LT and Gm-RT sequencing primers are indicated as arrows. Adapted from Ref. 5.

2.2. Analysis of Transconjugates with PhoA Activity: Cloning of the DNA Flanking Mini-*OphoA* Integrations and DNA Sequencing

Restriction endonucleases and T4 DNA ligase were obtained from Gibco-BRL (Rockville, MD, USA) or New England Biolabs (Mississauga, ON, Canada) and were stored at -20°C and used as per manufacturer's instructions.

3. Methods

The following procedures have been optimized for use with *Burkholderia* spp. It may be necessary, however, to modify some of the steps described below in order to achieve optimal results in other organisms.

3.1. Conjugation and Screening

1. Day 1: Inoculate an LBGMXP plate with SM10 (pmini-*OphoA*) from a frozen glycerol stock. Additionally, inoculate an LBSm plate with the recipient bacterial strain. Be sure to streak for isolated colonies. Invert and incubate plates at 37°C overnight.
2. Day 2: Using a sterile toothpick, inoculate 2 mL of LBGM broth in a snap cap tube with a single white SM10 (pmini-*OphoA*) colony. Again, using a sterile toothpick, inoculate 2 mL of LBSm broth in a snap cap tube with a single colony of the recipient bacterial strain and. Incubate at 37°C with aeration (250 rpm) for 18 h.
3. Day 3: Divide an LB agar plate into eight sections with a marker, label one section as the donor control and one section as the recipient control, label the other six sections with an "X" for the donor plus recipient conjugations (see Fig. 4). Pipet 5 μ L from the overnight culture of SM10 (pmini-*OphoA*), i.e., the donor, onto the donor control section and 5 μ L onto each of the "X" sections of the LB agar plate. Next, pipet 5 μ L from the recipient strain overnight culture onto the recipient control section and onto each of the "X" sections of the agar plate. Make sure that the cultures spotted onto to the "X" sections are mixed. Incubate the plate at 37°C overnight. For alternate conjugation methods (see Note 4).
4. Day 4: Using a sterile scraper or glass spreader, scrape the cells from each conjugation ("X" section) and spread them onto selective media, LBSmGmXP. Additionally, scrape the cells from the control sections onto selective media. Incubate at 37°C for 24–48 h.

5. Days 5 and 6: Examine LBSmGmXP plates for the presence of transconjugates. Blue (PhoA+) colonies represent transconjugates that have acquired mini-*OphoA* and have 'phoA' fusions. Retain the PhoA+ colonies for further analysis. There should not be any growth present on the control plates.
6. Purify the PhoA+ colonies by streaking them onto LBSmGmXP plates to ensure a homogeneous culture. At this point, it is suggested that frozen glycerol stocks be prepared by adding saturated bacterial culture to 40% glycerol in a 1:1 ratio, store at -70°C. The PhoA+ colonies for further analysis should be maintained on selective media.

3.2. Analysis of Transconjugates with PhoA Activity: Cloning of the DNA Flanking Mini-*OphoA* Integrations and DNA Sequencing

1. Inoculate a single PhoA+ transconjugate colony into 3 mL of LBSmGm in a snap cap tube. Incubate overnight at 37°C with aeration (250 rpm).
2. Isolate the chromosomal from the overnight cultures of each PhoA+ transconjugate using the method of your choice, we use the WizardTM Genomic DNA

Mini-*OphoA* Mutagenesis

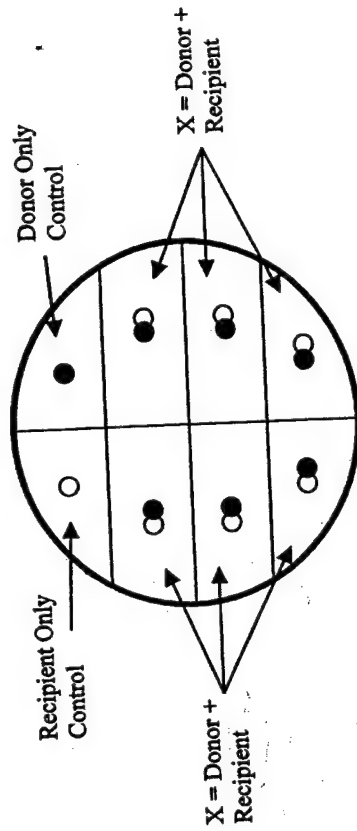


Fig. 4. Agar plate conjugation procedure used for transfer of mini-*OphoA* from SM10 λ pir (donor strain) to a gram negative bacterial recipient strain. Two control sections containing 5 μ L of donor or recipient strain alone, and six conjugation ("X") sections containing 5 μ L of both donor and recipient strains mixed together are shown.

Isolation kit (Promega). Quantitate the concentration of the chromosomal DNA obtained using OD260/280 method (8).

3. Self-cloning of the DNA flanking mini-*OphoA* integrations: digest 1–2 μ g of chromosomal DNA from each PhoA+ strain with an appropriate restriction endonuclease. For example, to clone the 'phoA' fusion joint, *NotI*, *SwaI*, *PacI*, *SpeI* or *Ascl* can be used. Alternatively, to clone the DNA flanking the opposite (Gm^R gene) side of mini-*OphoA*, *SfiI*, *FseI*, *Ascl*, *SaII*, *AvrII*, *PvuII*, or *NsiI* can be used (see Fig. 2). Set up a 20 μ L restriction endonuclease reaction in a 1.5 mL microfuge tube as per manufacturer's instructions. Generally, incubation at 37°C for 1 h is appropriate.
4. Heat inactivate the restriction digest by boiling for 5 min (see Note 5). Briefly centrifuge to recover any condensate.
5. Ethanol precipitate the DNA. Add 1/10 volume 3 M NaOAc, pH 4.6, and 2.5 vol of ice cold 100% Absolute ethanol. Place this reaction at -20°C for at least 30 min (see Note 6). Centrifuge at top speed in a microfuge for 15 min. Carefully remove the supernatant and wash pellet with 70% ethanol. Centrifuge for 5 min. Carefully remove the supernatant and air dry the pellet.
6. Set up a ligation reaction as follows. Thoroughly resuspend the dried DNA pellet from step 5 in 50 μ L of sterile deionized water (see Note 7). Use 19 μ L of the resuspended DNA, 5 μ L of 5X ligase buffer and 1 μ L of T4 DNA ligase (Gibco-BRL). Incubate at 16°C overnight.
7. Use 2–5 μ L of the ligation mixture from item 6 to transform high efficiency competent *E. coli* cells (see Note 8). For chemical transformations, we use *E. coli* Top 10 cells (Invitrogen) and for electroporations, we use Max Efficiency *E. coli* DH5 α cells (Gibco-BRL) as per manufacturer's instructions. Select for trans-formants on LBGMXP plates, incubate overnight at 37°C.

8. Using a sterile toothpick, inoculate individual blue transformants into LBGM broth. Incubate at 37°C with aeration (250 rpm).
9. Isolate plasmids from each transformant using your method of choice, we use the QIAprep plasmid miniprep kit (QIAGEN). Check each plasmid by digesting with the same enzyme that was used to clone it. Load the digested plasmids onto a 0.8% agarose gel. The appropriate clones will have only one band when visualized with ethidium bromide under a UV light source. The plasmid size can then be estimated; additional double digestions can be performed to more accurately determine the size of the cloned flanking DNA fragment.
10. Sequence the appropriate plasmids using the Pho-LT and Gm-RT primers (see Note 9).
11. Analyze the sequence obtained for homology to known gene sequences using the BLASTX program.

4. Notes

1. Gentamicin is used to select for the presence of mini-*OphoA* in the donor strain prior to conjugation, and to select for the integration of mini-*OphoA* into the chromosome of the recipient strain following conjugation. If Gm is not a desirable selectable marker for a specific recipient bacterial strain, the Gm^R cassette on the mini-*OphoA* can easily be replaced. This cassette can be excised using *SacI* or *SrfI* and a resistance cassette of the user's choice can be ligated into mini-*OphoA*.
2. Streptomycin is used for selection against the donor strain, SM10 λ pir (mini-*OphoA*), following conjugations procedures. When using certain gram negative bacterial strains, it may be necessary to use an antibiotic other than Sm if the recipient strain does not display a streptomycin resistant phenotype. For example, when *B. mallei* was used as a recipient for mini-*OphoA*, naladixic acid was used in place of Sm due to the fact that a stable Sm^R derivative of *B. mallei* could not be obtained (5,7).
3. Low salt Luria-Bertani broth (10 g tryptone, 5 g yeast extract and 5 g NaCl, not 10 g NaCl) was used throughout this protocol as high salt concentrations may interfere with the activity of the gentamicin.
4. The conjugation method described in Subheading 3.1., item 3 has been used specifically for *B. pseudomallei* and *B. thailandensis*. For other gram negative bacteria, the incubation time at 37°C may need to be adjusted depending on the conjugation efficiency of the recipient strain. Additionally, the bacterial growth from the conjugations can be scraped off of the LB plate and diluted as necessary in 0.85% NaCl and then plated. This step may be taken if the density of single transconjugates on the selective media is too high. Other methods of conjugation may be used instead of using the plate method described here. A broth method may be employed as follows: inoculate a snap cap tube containing 2 mL LB broth with 100 μ L of overnight cultures of each of the donor and the recipient strains, incubate at 37°C 250 rpm for a few hours to overnight. Following the incubation, 100 μ L aliquots (or less if necessary) of the conjugation mixture should be plated

on selective media (LBSmGmXP) and incubated at 37°C. The duration of the conjugation step may differ for different bacterial species and should be optimized for the specific gram negative recipient strain in question.

5. The heat inactivation step may be altered depending on the restriction endonuclease used, for example heating to 65°C for 10 min is sufficient for inactivation of certain restriction endonucleases. See the manufacturer's heat inactivation specifications for the particular enzyme being used.
6. During the ethanol precipitation step, we have found that placing the reaction in a -20°C or -70°C freezer overnight works efficiently, and may in fact increase the amount of DNA recovered following this step.
7. It is important at this stage to ensure that the dried DNA pellet is thoroughly resuspended. It is suggested that the DNA be allowed to resuspend at room temperature for at least 10 min prior to preparation of the ligation reaction.
8. The use of high efficiency competent *E. coli* cells was necessary to obtain mini-*OphoA* flanking clones. User prepared cells did not have a high enough transformation efficiency to obtain clones on the first cloning attempt. Additionally, it is often helpful to microconcentrate the ligation reactions prior to transformation in order to increase the chances of obtaining the desired transformants.
9. Nucleotide sequencing was performed with the ABI PRISM DyeDeoxy Termination Cycle Sequencing System and analyzed using an ABI 1373A DNA Sequencer by University Core DNA Services (University of Calgary). Both the Pho-LT and Gm-RT primers can be used for sequencing of plasmid DNA isolated from a single mini-*OphoA* flanking clone regardless of which side of the integration was cloned. For example, if the *phoA* fusion joint was cloned, PhoA-LT would provide the sequence immediately adjacent to *phoA*, while the Gm-RT primer would provide the sequence of the DNA upstream. Alternately, if the Gm^R gene joint was cloned, Gm-RT would provide the sequence immediately adjacent to the integration and Pho-LT would provide the sequence of the DNA downstream of the mini-*OphoA* integration. This feature of self-cloning expedites the sequencing process and allows the user to quickly and efficiently assess the interrupted gene as well as neighboring genes.

Acknowledgments

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Flagellum-Mediated Adhesion by *Burkholderia pseudomallei* Precedes Invasion of *Acanthamoeba astronyxis*

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In this study we investigated the role of the bacterial flagellum in *Burkholderia pseudomallei* entry to *Acanthamoeba astronyxis* trophozoites. *B. pseudomallei* cells were tethered to the external amoebic surface via their flagella. MM35, the flagellum-lacking *fliC* knockout derivative of *B. pseudomallei* NCTC 1026b did not demonstrate flagellum-mediated endocytosis in timed coculture, confirming that an intact flagellar apparatus assists *B. pseudomallei* entry into *A. astronyxis*.

Burkholderia pseudomallei is the etiologic agent of melioidosis, a potentially fatal infection endemic in tropical Australia and southeast Asia (1). *B. pseudomallei* is a facultative intracellular bacterial pathogen with the capacity to invade a variety of cell types including macrophages, in which the bacteria can survive for prolonged periods (7). Escape from sequestered cellular locations is thought to explain recrudescence and delayed-onset *B. pseudomallei* infection (12). In a recent study it was shown that *B. pseudomallei* entry into macrophages results in cellular effects similar to those caused by other intracellular bacteria (8).

In common with other intracellular bacterial pathogens such as *Legionella pneumophila* and *Listeria monocytogenes* (9, 11), *B. pseudomallei* has been shown to enter and survive within free-living amoebae belonging to the genus *Acanthamoeba* (6). We noted previously that an unusual form of tethered bacterial motility occurred shortly after *B. pseudomallei* cells attached to the amoebic trophozoite (6), prompting the present investigation into whether or not adherence by *B. pseudomallei* flagella was required to initiate bacterial entry into the amoebic trophozoite.

Bacterial strains and growth conditions. The *B. pseudomallei* strains used in this investigation were NCTC 13177 (also known as WKO97, the cause of a small outbreak of melioidosis in Western Australia during 1997 [4, 5]), NCTC 1026b, and the flagellin-lacking mutant MM35, derived from NCTC 1026b (2). MM35 contains Tn5-OT182 integrated within the flagellin structural gene, *fliC*. The mutation is nonpolar and is complemented by cloned *fliC* in *trans* (2). These bacteria were stored in 20% glycerol at -70°C until required and were then recovered by culture onto fresh 5% horse blood agar at 37°C in air for 18 h. Five colonies were picked and used to inoculate 15 ml of fresh Trypticase soy broth (Excel Laboratory Products, Bentley, Western Australia) and incubated at 37°C in air for

18 h. A 1:10 dilution of this 18-h culture was then made in fresh Trypticase soy broth and incubated for a further 1 h 30 min in air at 37°C to obtain mid-lag-phase bacteria, as previously described (6).

Amoeba culture. *Acanthamoeba astronyxis* (CCAP 1534/1) was maintained in axenic form in PYG broth (Excel Laboratory Products) in tissue culture flasks at 28°C . An aliquot of supernatant was harvested from the culture flask immediately before coculture procedures and concentrated by centrifugation at $1,000 \times g$ for 5 min, and the supernatant was replaced with sterile 0.8% NaCl solution. The cell count was obtained by examining an aliquot of this suspension in a counting chamber (Fuchs-Rosenthal). This was adjusted by dilution to 10^5 cells/ml.

Coculture conditions. Coculture of *B. pseudomallei* with *A. astronyxis* was performed by adding the mid-lag-phase bacterial suspension to the amoebic suspension either in a small volume (1.0 ml) for light microscopy or in larger volumes (10 ml) in capped tubes for electron microscope preparations. In the timed-coculture experiment, NCTC 1026b and MM35 were set up in parallel as coverslip preparations. These preparations were examined alternately, 20 consecutive cells were viewed on each occasion, and features previously described (6) were noted. In view of the negative results obtained with MM35, a coculture preparation of this strain was centrifuged onto amoebic trophozoites ($3,000 \times g$ for 15 min) and examined after 60 min at 20°C . All aerosol-generating procedures were conducted in a class II biological safety cabinet.

Electron microscopy. One-, 5-, and 30-min coculture preparations were used for gold sputter coating. Ten-millimeter-diameter glass coverslips containing the organism were fixed in 2.5% glutaraldehyde in 0.05 M cacodylate buffer at pH 7.4 overnight, washed well in 0.05 M cacodylate buffer, postfixed in 1% aqueous osmium tetroxide, dehydrated through graded alcohol, and dried in a Polaron critical-point drying apparatus using superdry alcohol as the chamber liquid and liquid CO_2 as the exchange gas. They were mounted on aluminum stubs with carbon conductive adhesive tape and coated with a 15-nm layer of gold and palladium in a Polaron E5100 sputter coating unit. An E5500 quartz crystal monitor was used to control the thickness of the metal coat. The stubs were stored in a desiccating

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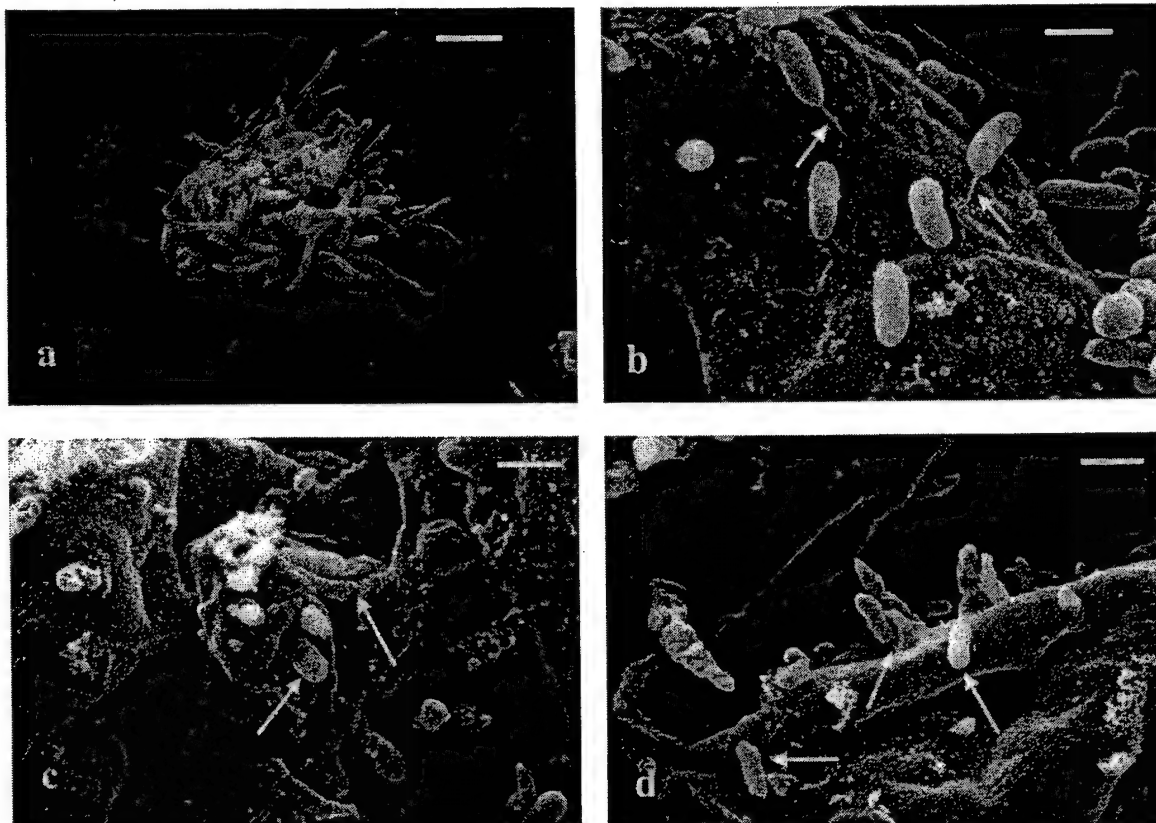


FIG. 1. Scanning electron microscope views of *B. pseudomallei* and *A. astronyxis* coculture. (a) *A. astronyxis* trophozoite prior to inoculation with *B. pseudomallei* NCTC 13177. Scale bar = 10 μ m. (b) Trophozoite surface covered with adherent *B. pseudomallei* NCTC 13177 cells, some of whose flagella (arrows) are visible. Scale bar = 2 μ m. (c) *B. pseudomallei* NCTC 13177 cells (arrows) tucked in folds of the trophozoite surface during phagocytosis. Scale bar = 2 μ m. (d) *B. pseudomallei* NCTC 13177 cells (arrows) remaining on the external trophozoite surface showing end-on adherence. Scale bar = 2 μ m.

chamber containing silica gel until viewed in a XL30-TM scanning electron microscope (Philips, Eindhoven, The Netherlands).

Scanning electron microscopy of cocultures. Scanning electron microscopy of *A. astronyxis* trophozoites demonstrated an external surface covered with tufts of filamentous pseudopodia or filopodia (Fig. 1a). In coculture preparations NCTC 13177 bacilli were found on the external trophozoite surface. Many bacilli were observed to demonstrate end-on adherence (Fig. 1b), and in several a thickened flagellum tethering the bacillus to the trophozoite external surface could be seen (Fig. 1c). Individual bacilli were also seen in clefts between extensions of the trophozoite surface (Fig. 1d). NCTC 1026b exhibited the same series of interactions with *A. astronyxis* (results not shown).

Phase-contrast microscopy of coculture. In a timed coculture, NCTC 1026b followed the sequence of cellular events noted previously for other strains of *B. pseudomallei* (6), including flagellar adherence, tethered motility, incorporation into single-bacillus vacuoles, and formation of tufts of bacilli on the trophozoite surface (Table 1). Tethered motility was inferred from the high-frequency rotation of a bacillus around a fixed point on the external amoebic surface in a helicopter blade-like motion. Bacillary tufts were noted when a cluster of five or more bacilli were observed in parallel alignment with each other, all adhering end-on to the amoebic surface. The flagellin-negative derivative

MM35 did not demonstrate any of these phenomena during the period of observation in the series of five repeat experiments (Table 1). During the first 60-min observation, MM35 was observed in single-bacillus vacuoles in 5 of the 100 trophozoites examined. No multibacillary vacuoles were seen. When the MM35 preparation was examined after a 24-h incubation, intracellular bacilli were noted in unibacillary vacuoles in only 7 of the total of 100 trophozoites examined (five preparations, each of 20 cells). Only two multibacillary vacuoles were seen at 24 h, and each of these contained only two bacilli. Repetition of the coculture with MM35 precipitated onto optimally receptive *A. astronyxis* trophozoites by centrifugation did not increase uptake over a 60-min observation period.

The role of the *B. pseudomallei* flagellum in adherence to phagocytic eukaryotic cells was inferred from a previous subjective observation (6). These preliminary observations were insufficient to establish that the flagellum of *B. pseudomallei* was necessary for incorporation in an amoebic endocytic vacuole and did not establish a causal relationship between bacterial adherence and eukaryotic cellular invasion.

The results of the present study provide more-direct evidence for the involvement of the *B. pseudomallei* flagellum in eukaryotic cellular invasion. The striking difference in amoeba-invasive capacity between *B. pseudomallei* strain NCTC 1026b and the corresponding flagellin-negative motility mutant, MM35,

TABLE 1. Cellular outcomes of *B. pseudomallei* cocultured with *A. astronyxis*

<i>B. pseudomallei</i> strain	Time (min)	In amoebic trophozoites ^a no. of:						
		Adherent bacilli ^b	Rotating bacilli ^c	Single-bacillus vacuoles ^d	Multibacillary vacuoles ^e	Bacillary tufts ^f	Bacilli in chains ^g	Bacillary tangles ^h
NCTC 1026b	0	9.4 ± 4.83	3.8 ± 3.6	0.8 ± 0.8	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	20	11.8 ± 3.35	5.2 ± 1.3	7.2 ± 2.6	10.4 ± 4.4	0.6 ± 0.9	0.00 ± 0.00	0.00 ± 0.00
	40	10.4 ± 2.9	5.0 ± 1.4	4.8 ± 1.3	12.8 ± 2.8	0.8 ± 0.8	0.00 ± 0.00	0.00 ± 0.00
	60	6.4 ± 3.0	1.8 ± 1.6	4.0 ± 0.7	13.6 ± 2.3	0.6 ± 0.9	0.00 ± 0.00	0.00 ± 0.00
	1,440	17.4 ± 3.1	15.4 ± 5.1	2.4 ± 2.2	6.2 ± 2.6	5.2 ± 2.7	2.2 ± 2.0	1.6 ± 3.0
MM35	0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	20	0.00 ± 0.00	0.00 ± 0.00	0.20 ± 0.45	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	40	0.00 ± 0.00	0.00 ± 0.00	0.80 ± 0.45	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	60	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	1,440	0.20 ± 0.45	0.00 ± 0.00	1.40 ± 1.52	0.40 ± 0.90	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

^a At each time interval, 20 amoebic trophozoites were examined by phase-contrast microscopy for the presence of each feature indicated. Each series of observations was repeated with a total of five complete cocultures for both *B. pseudomallei* strains. Values shown are the means ± standard deviations of the numbers of trophozoites exhibiting each feature from five consecutive replicate experiments.

^b Bacilli adhering to the trophozoite surface via the bacillus end.

^c One or more bacilli in tethered high-frequency rotation around a point on the external trophozoite surface.

^d One or more trophozoite vacuoles containing single bacilli.

^e One or more trophozoite vacuoles containing more than one distinct bacillus.

^f Five or more bacilli in a single tuft of parallel bacilli adhering to the external trophozoite surface.

^g Five or more bacilli in one or more chains of bacilli adherent to the external trophozoite surface.

^h Bacilli in a tangled web of chains external to the trophozoite or cyst surface.

supports a critical role for the flagellum in the early stages of cellular invasion. The lack of motility in MM35 reduces the normally observed frequency of encounter between rapidly motile, mid-lag-phase *B. pseudomallei* and receptive *A. astronyxis* trophozoites, but centrifugation of bacteria onto the amoebae did not improve bacterial uptake. The comprehensive lack of other cellular events such as formation of vacuoles containing bacilli, bacterial escape, and tuft formation with strain MM35 indicates that tethered motility is the first in a series of interactions between *B. pseudomallei* and *A. astronyxis*. Further study of flagellum-mediated adherence by *B. pseudomallei* may therefore assist in the selection of suitable targets for subsequent development of antiadhesion vaccine candidates.

We note with interest a recent report that *Aeromonas caviae* requires motility and flagellar function to maximize adherence to human epithelial cells (10). Other bacterial pathogens appear to employ a flagellar-adhesion strategy. In a recently published report, a nonflagellate *Legionella pneumophila* flaA mutant was used to demonstrate that the *Legionella* flagellum was required for invasion of HL-40 cells and *Acanthamoeba castellanii* trophozoites but not for adherence (3). That study centrifuged bacteria onto the amoebic cells, a procedure that may have obscured bacterial adherence that depends on delicate structures such as flagellar tips. Nevertheless, their results imply a cellular interaction different from that between *B. pseudomallei* and *A. astronyxis*, in which flagellar adherence is a necessary precursor to invasion. Detailed comparison of these two bacterium-eukaryote pairs may provide useful insights into the cellular mechanisms involved.

In conclusion, our investigation of the interaction between *B. pseudomallei* and *A. astronyxis* trophozoites supports a critical role for the bacterial flagellum in the early stages of cellular invasion. Further work is needed to characterize the na-

ture of the eukaryotic cell surface receptor, the signaling events that trigger cytoskeletal rearrangements in the amoeba, the molecular basis of this interaction, and the extent of parallels with *B. pseudomallei*-mammalian phagocytic cell systems. The *B. pseudomallei*-*A. astronyxis* system provides a useful model with which to explore the cellular pathogenesis of melioidosis.

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The use of animal infection models to study the pathogenesis of melioidosis and glanders

Donald E. Woods

The use of animal infection models is central to the study of microbial pathogenesis. In combination with genetic, immunological and antigen purification techniques, much can be learned regarding the pathogenesis of diseases caused by microorganisms. This update focuses on the recent use of animal infection models to study the pathogenesis of melioidosis and glanders.

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The long-term objective of our research is to define at a molecular level the pathogenesis of diseases caused by *Burkholderia mallei* and *Burkholderia pseudomallei*, bacterial pathogens that cause the diseases glanders and melioidosis, respectively [1]. Based on biochemical, immunological and genetic data, *B. pseudomallei* and *B. mallei* are closely related species in the genus *Burkholderia*. As *B. mallei* and *B. pseudomallei* are of significance as agents of bioterrorism (listed as Category B by the CDC) and biological warfare, the development of effective vaccines and treatments is of particular concern. Our understanding of the diseases caused by these organisms is rapidly improving, and we are moving forward with our studies on the pathogenesis of these diseases with a view to developing new and effective vaccines and/or therapies against these organisms.

Glanders and melioidosis

Glanders is a zoonosis, and humans whose occupations put them into close contact with infected animals can contract the disease. *B. mallei* is a host-adapted pathogen that does not persist in nature outside of its host, the horse [2]. There have been no naturally occurring cases of glanders in North America in the past 60 years but laboratory workers are still at risk of infection with *B. mallei* via cutaneous [3] and inhalational [4] routes. Human glanders has been described as a painful and loathsome disease from which few recover without antibiotic intervention [5,6]. Little is known about

the virulence factors of *B. mallei*, but a recent report indicated that the capsular polysaccharide is essential for virulence in hamsters and mice [7]. At present, no effective vaccines are available against *B. mallei*, and information on the treatment of infections with this organism by antibiotic therapy is sparse.

B. pseudomallei is a common cause of human pneumonia and fatal bacteremias in endemic areas [8]. This organism is an opportunistic pathogen, and those individuals who are particularly susceptible to *B. pseudomallei* infection have underlying conditions such as diabetes or renal disease [8,9]. The clinical manifestations of *B. pseudomallei* infection, a disease known as melioidosis, vary greatly from an asymptomatic state to benign pneumonitis, acute or chronic pneumonia or to overwhelming septicemia [10]. Treatment of melioidosis can involve up to nine months of antibiotic therapy [11], and relapse is common. Additionally, the latency period of *B. pseudomallei* infection can vary from two days to 26 years [10,12].

Reports in the literature suggest that there are *B. pseudomallei*-like species that are avirulent in animals [13–15]. We cloned and sequenced >95% of the 16S rDNA from both *B. pseudomallei* 1026b, a virulent clinical isolate, and *B. pseudomallei*-like E264, an avirulent environmental isolate, to assess definitively the relatedness of these organisms to each other. The results of these studies, based on phylogenetic analysis of 16S rDNA, confirmed the presence of a new *Burkholderia* species, which we named *Burkholderia thailandensis* [16].

Detecting virulence genes and animal models

The detection of virulence genes in bacteria is of great biological interest. We have developed a method that combines PCR-based subtractive hybridization, insertional mutagenesis and an animal infection model to detect virulence genes efficiently, and have applied this method to *B. pseudomallei* and *B. mallei*. Virulence genes specific to these pathogens were

identified by subtractive hybridization against the related non-pathogenic species *B. thailandensis*. An internal, AT-rich fragment of one of the *B. pseudomallei* genes was cloned into a mobilizable suicide vector that allowed the site-specific insertion of this fragment into the wild-type gene in *B. pseudomallei*, thus creating a specific knock-out mutation that rendered this strain avirulent in a hamster infection model for melioidosis [17]. The phenotype of this mutant, *B. pseudomallei* SR1015, is similar to that of *B. thailandensis*. Specifically, neither *B. pseudomallei* SR1015 nor *B. thailandensis* produces a major surface capsular polysaccharide, and both organisms have attenuated virulence in hamsters relative to wild-type *B. pseudomallei* [18]. Following these observations, we determined that the capsular polysaccharide is indeed present in *B. mallei*, and that the capsule is important for *B. mallei* virulence in a hamster infection model for glanders [7,19].

Outbred strains of mice have also been used as models of melioidosis and glanders [20,21], and information obtained from these studies has provided additional understanding of the pathophysiology of these diseases. A novel approach to studying the pathogenesis of *Burkholderia* spp. using an invertebrate model, *Caenorhabditis elegans*, was developed by Jeddalo and colleagues [22]. Unfortunately, this model appears to show little correlation with virulence in vertebrates as *B. thailandensis*, which is avirulent for humans, kills *C. elegans* readily. Recently, Gan and colleagues reported on the use of *C. elegans* to study the pathogenesis of *B. pseudomallei* and *B. mallei*, and obtained very different results from those of Jeddalo, particularly as Gan *et al.* [23] reported that *B. thailandensis* is avirulent in the *C. elegans* model, and *B. mallei* is virulent. Gan and colleagues attribute the differences in observations between the two studies to possible differences in the strains used in the two separate studies; however,

Melioidosis vaccines

Jonathan Warawa and Donald E Woods[†]

Melioidosis is a disease caused by the facultative intracellular pathogen *Burkholderia pseudomallei* and is associated with a high mortality rate. Melioidosis is endemic in the tropics of southeast Asia and northern Australia and is of worldwide concern, particularly as it is a potential agent of bioterrorism or biological warfare. Also of concern is the lack of a fully effective antibiotic regime, as cases of bacteremia have unacceptably high mortality rates and relapse of melioidosis is common. This review focuses on the approaches that have been undertaken towards the development of an effective vaccine against this disease and highlights current strategies being used to move towards finalizing such a vaccine.

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Disease

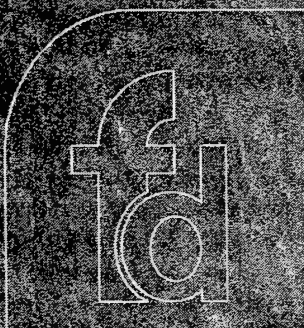
Melioidosis is a multifaceted disease caused by the Gram-negative bacterial pathogen *Burkholderia pseudomallei*. Melioidosis is endemic primarily in the tropics of southeast Asia and northern Australia. However, melioidosis is not geographically isolated and it is considered to be a potential agent of biological warfare, making it a disease of worldwide concern [1]. The symptoms of melioidosis may vary with the route of infection, but generally all forms include flu-like symptoms of fever, headache, chills and joint pains. Melioidosis can manifest as pneumonia when acquired through inhalation, or as skin abscesses if introduced through skin penetration [2]. Additional routes of infection are possible, such as through sexual transmission, injection, or mother-to-child transmission across the placental barrier [3,4].

Melioidosis can lead to spleen, liver and brain abscesses, as well as osteomyelitis, all of which eventually give rise to a highly fatal bacteremia (50% mortality with treatment) [2]. Melioidosis can also exist as a chronic disease, correlated with the intracellular nature of *B. pseudomallei* [5]. Additionally, reports of latency for up to 29 years post exposure have been observed [6].

Epidemiology

Melioidosis is a disease primarily found in southeast Asia and northern Australia and it is one of the most common causes of fatal community-acquired bacteraemic pneumonia in northern Australia [7]. Indeed, pneumonia is the most common manifestation of melioidosis, contributing to approximately 50% of reported cases [2]. *B. pseudomallei* is a soil bacterium that can be readily cultured from the water and soil of rice paddies and oil palm fields and it is also found in stagnant pools during the rainy season [8]. Infection can therefore occur through the subcutaneous inoculation of bacteria through injury and manifest as skin abscesses (~18% of cases [2]).

The physical well being of an individual can influence their susceptibility to infection with *B. pseudomallei*. Risk-factors associated with an increased susceptibility include diabetes, excessive alcohol consumption, chronic lung or renal disease and over-consumption of kava (native Australian beverage made from pepper plant root) [2]. In northern Australia, men have been found to be at higher risk of infection than were women or children, although this is probably due to the higher incidence of farming-related professions filled by males in endemic areas [9].



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vaccine, DNA vaccine, glanders,
heterologous vaccine, melioidosis

Treatment

B. pseudomallei infection requires specialized antibiotic treatment, as this organism is resistant to many commonly used antibiotics. The inability to correctly diagnose and administer the correct antibiotics may lead to bacteraemia, which has a mortality rate of 74% with conventional treatment [10]. Therefore, effective treatment of melioidosis first requires successful diagnosis or suspicion of the disease. Ultimately, culture of *B. pseudomallei* is the only conclusive evidence of infection. The current consensus is that the most effective antibiotic(s) for treatment of melioidosis is ceftazidime or imipenem, resulting in the mortality of approximately a third of patients with severe melioidosis [10–15]. Follow-up maintenance oral therapy should be maintained for 20 weeks, as *B. pseudomallei* remains intracellularly for extended periods of time and relapse is possible in a quarter of infected individuals [16]. However, maintenance therapy of cotrimoxazole and doxycycline for 20 weeks is reported to reduce the relapse rate to 3% [17].

Initial treatment with granulocyte colony-stimulating factor (G-CSF) was found to significantly increase the survival rate of patients suffering from *B. pseudomallei*-induced septic shock. The cost of G-CSF may be prohibitively expensive and more trials are required [18]. In contrast, inhibition of G-CSF in mice did not have a major effect on the virulence of *B. pseudomallei* [19].

Rationale for vaccine development

B. pseudomallei is an organism that is difficult to treat using antibiotics. Antibiotics must be administered over extended periods of time and relapse is possible. In addition, lack of efficient diagnosis to treat the disease early in the infection can lead to a much more fatal bacteraemic form of melioidosis. Therefore, conventional therapies yield an unacceptably high mortality level making vaccination desirable to inhabitants of endemic areas.

Of current interest is the prospect of human pathogens being used as agents of bioterrorism. *B. pseudomallei* is a potential candidate for use in bioterrorism, due to its high mortality rate and flexibility of routes of infection – most importantly through inhalation. As antibiotic treatments are not fully effective and must be used in high quantities, a widespread biological attack with this pathogen would have severe consequences. A melioidosis vaccine would benefit military personnel at risk from biological attack, while it is not likely that a large-scale immunization regime of all 'Western' nation inhabitants would be contemplated, especially as *Bacillus anthracis* and the smallpox virus are the more likely candidates for use in biological warfare.

Pathogenesis of melioidosis

Virulence factors & protective antigens

We are beginning to understand the molecular processes that direct the pathogenicity of *B. pseudomallei*. It is known that *B. pseudomallei* is an invasive pathogen that resides within a vacuole in eukaryotic cells [20,21], although it has also been demonstrated that *B. pseudomallei* can break out of this vacuole to enter the cytoplasm [22]. The intracellular nature of *B. pseudomallei* makes

treatment and vaccination against this pathogen much more difficult. Understanding the mechanisms by which *B. pseudomallei* exerts its pathogenicity upon its host is key to developing effective vaccines against this pathogen. The recent advent of the completed *B. pseudomallei* genome sequencing project at the Sanger Institute (Cambridge, UK) will no doubt accelerate this research.

Secreted antigens

B. pseudomallei secrete numerous proteins into culture supernatant, including two thermolabile toxins [23,24], several proteases [25–27], phospholipase C [28], hemolysin, lecithinase and lipase [29]. Several of these enzymes are dependent upon the general secretory pathway machinery for their release, presumably in conjunction with a Type II secretion apparatus [30].

In addition, it has recently been found that *B. pseudomallei* possesses at least three different Type III secretion systems (TTSSs), each of which is predicted to be activated under specific conditions to deliver virulence factors into target cells [31–33]. The function of each of these three TTSSs has not yet been elucidated. However, TTSS islands in related pathogens deliver effector molecules to facilitate such functions as the invasion and vacuole survival phenotypes observed for *B. pseudomallei*. Indeed, one *B. pseudomallei* TTSS island (TTSS3) possesses homology to the SPI-1 pathogenicity island from *Salmonella enterica* required for host cell invasion [31]. *Salmonella*, which also resides in a vacuole in host cells, has a second TTSS island that is required for survival of the bacterium in the vacuole [34]. Although *B. pseudomallei* has no homologous TTSS to the *Salmonella* SPI-2 island, it is possible that one of the additional *B. pseudomallei* TTSS islands (TTSS1 or TTSS2) might fill this role.

Any proteins secreted/released by these mechanisms could be recognized by the immune system and therefore serve as bases for vaccine development. However, priming the immune system against such targets would not entail targeting of the immune system directly against the pathogen itself, which would not be desirable. Conversely, putative *B. pseudomallei* effector molecules required for the persistence of *B. pseudomallei* in a host cell may be released into the host cell cytoplasm and subsequently become presented to the immune system. If immune system priming against such a molecule were available, it may be possible to arrest the chronic state of disease associated with melioidosis. However, a significant amount of research remains to be completed in order to identify such putative effector molecules.

Cell-associated antigens

Cell-associated antigens are likely to be the best vaccine candidates as the pathogen itself becomes a target. *B. pseudomallei* components that may serve as effective targets for the immune system include lipopolysaccharide (LPS), capsule, flagella and pili. Characterization of *B. pseudomallei* pili has not been published, however, a gene cluster putatively encoding a Type IV pilus was recently identified, suggesting that this structure is indeed present [33]. Conversely, LPS (O-PS II), capsular polysaccharide (CPS; formerly O-PS I) and flagellin proteins

have all been initially characterized and found to be immunogenic in melioidosis patients [35–38]. In addition, antibodies raised against LPS and flagellin have provided passive protection against infection by *B. pseudomallei* [35,39,40].

Vaccination strategies

Vaccine development for melioidosis is made difficult in that *B. pseudomallei* is an intracellular pathogen that can effectively hide from the immune system for extended periods of time. Effective vaccination against intracellular pathogens therefore requires the elicitation of a cytokine-mediated cellular response (Th1 type response) so as to combat potential recurrence of disease. Indeed, a host response against *B. pseudomallei* requires interferon (IFN)- γ , tumor necrosis factor (TNF)- α and interleukin (IL)-12 cytokines in order to combat disease in a murine model, indicative of a Th1 response [19]. However, it has also been reported that Th2 cytokines are also expressed such that the immune system is not wholly polarized to either a delayed-type hypersensitivity (Th1) or a neutralizing antibody response (Th2) [41]. It was also demonstrated that a careful balance of cytokine production is required, as overproduction can lead to a rapid acute form of melioidosis [41]. Therefore, it may not currently be possible to predict the efficacy of *B. pseudomallei* vaccines that favor either a Th1- or Th2-type response. Described below are a number of approaches that have been used to generate a *B. pseudomallei* vaccine.

Conjugate vaccines

Often, a conjugation between a carrier molecule and a lesser immunogenic molecule(s) is generated, whereby the properties of the carrier protein direct an immune response against the weakly immunogenic molecule. Alternatively, conjugation of molecules can enhance an immune response against that antigen(s) due to the generation of a larger complex structure for the immune system to recognize. The latter strategy was used to generate a significant response against the *B. pseudomallei* antigens flagellin, CPS and O-PS [42]. Each of these antigens were known to be individually immunogenic and sera raised against these antigens provided passive protection against *B. pseudomallei* infection [39,40], yet a conjugate of these antigens successfully resulted in a strong immunogenic response against capsule (CPS), O-PS (LPS) and flagellin [42].

Unfortunately, this conjugate could not be tested as a vaccine, as no suitable animal model was available at that time, whereby control animals would still be susceptible to *B. pseudomallei* infection following the time span of an immunization regime. However, antiserum raised against the polysaccharide (PS)-flagellin conjugate was successfully used to induce passive protection in diabetic infant rats against subsequent infection by *B. pseudomallei* [42]. This suggests a potential future for the use of conjugate vaccines in the treatment of melioidosis.

A closely related pathogen, *Burkholderia mallei*, the causative agent of glanders, is also a subject of study in our laboratory with respect to vaccine development. *B. mallei* has a strong propensity for infection of equines, and horses have been a historical vector

for the transmission of glanders to humans. Thus, horses are ideal animals for the study of glanders. The CPS and O-PS structures (capsule and LPS) of *B. pseudomallei* and *B. mallei* are immunologically related, as antibodies generated against the CPS and O-PS of one strain cross-react with the other [43]. In addition, structural studies of the O-PS from LPS are shown to be almost identical between the two strains, except that *B. mallei* LPS lacks an O-acetyl group on the talose residue [44]. The genes required for the generation of capsule in *B. pseudomallei* and *B. mallei* were also shown to be virtually identical, supporting the close relation between the CPS structures of these pathogens [43,45].

We are currently investigating the use of a protein-polysaccharide (PPS) conjugate vaccine in a horse model of glanders. The conjugate consists of the CPS and O-PS molecules from *B. mallei* conjugated to exotoxin A of *Pseudomonas aeruginosa*, which is used as a carrier molecule to facilitate an immune response [46]. We have reason to believe that such a conjugate will be efficacious against glanders in horses and may therefore become a glanders vaccine for use in humans. Due to the relatedness of the CPS and O-PS molecules between *B. pseudomallei* and *B. mallei*, we predict that this vaccine will also provide protection against melioidosis in humans. If this conjugate becomes licensed for human use against glanders, we will then proceed to human trials in areas endemic for melioidosis.

DNA vaccines

Vaccination by means of a mammalian expression vector carrying the bacterial gene of interest is a novel approach to facilitate prolonged exposure of an antigen to the immune system. In addition to the potential longevity of the immune response, DNA vaccines tend to result in a Th1-type response to the encoded antigen, which may be desirable for immunoprophylaxis against melioidosis. DNA vaccines have the added benefit of being relatively inexpensive to manufacture and store. These factors make DNA vaccines an attractive mechanism for vaccinating against *B. pseudomallei*.

It is known that *B. pseudomallei* flagellin is immunogenic in patients with melioidosis, therefore, a DNA vaccine was recently generated using the *fliC* flagellin structural gene [47]. This gene was incorporated into a suitable mammalian expression vector and injected intramuscularly into either Syrian golden hamsters or diabetic rats. The DNA-immunized hamsters were not protected from *B. pseudomallei* following four immunizations, although they did develop an immune response to flagellin and began to express IFN- γ in the spleen, suggestive of a Th1 response. It is thought that the hamsters succumbed to disease due to their extreme sensitivity to melioidosis, although it may be possible to modify this vaccination strategy to develop immunity.

Diabetic rats are only susceptible to melioidosis during infancy, therefore, they were only immunized twice before challenging with *B. pseudomallei* infection. Immunized rats showed a statistical improvement in resisting disease compared with their nonimmunized counterparts [47]. Thus, there is hope that this alternative strategy could be optimized

to provide complete protection or used in conjunction with additional DNA molecules to maximize the potential of the immune system to recognize *B. pseudomallei*.

Attenuated vaccines

The classical approach to vaccination has been to protect an individual using a nonpathogenic variant of the pathogen in question. The immunogenic *B. pseudomallei* CPS was the subject of two independent mutagenesis trials that found that this structure is critical to the pathogenesis of *B. pseudomallei* [45,48]. Capsule mutants were also examined for potential as vaccines, though it was found that no protection was provided against subsequent challenge in a mouse model of infection [48].

Protection studies were also performed in mice using *B. pseudomallei* human clinical isolates of reduced virulence (mouse LD₅₀ > 10⁶) to immunize against subsequent challenge with a more virulent clinical isolate (mouse LD₅₀ < 10) [49]. A significant improvement in survival rates was demonstrated, suggesting that attenuated vaccines are feasible for melioidosis vaccination. However, it would not be desirable to inoculate a clinical isolate capable of inducing death into humans as a vaccine for melioidosis, therefore, this study demonstrated a proof of principle for melioidosis vaccines rather than a clear path forward. The use of an attenuated vaccine requires that the vaccine is not in any way lethal to the vaccine recipient and therefore additional studies would be required before the use of such a vaccine could be seriously entertained.

Recently, an auxotrophic *B. pseudomallei* mutant was found to be attenuated for virulence in mice and provided protection against subsequent challenge with a virulent *B. pseudomallei* strain [50]. This report confirms that attenuated vaccines are a viable approach to *B. pseudomallei* immunoprophylaxis and will no doubt receive more attention in the future.

Heterologous vaccines

A recent study has demonstrated that a nonpathogenic relative of *B. pseudomallei*, *B. thailandensis*, may be useful for vaccination against melioidosis [51]. Ilyukhin and colleagues demonstrated that vaccination with strains of *B. thailandensis* resulted in approximately 50% protection against subsequent *B. pseudomallei* challenge in a guinea-pig model of infection. With optimization, this may be a viable method to immunize against melioidosis. Similarly, Russian studies have demonstrated that vaccines against tularemia, plague and salmonellosis also to provide a degree of protection against melioidosis in white mice by increasing the LD₅₀ by a log factor and moderately increasing life expectancy [52].

Expert opinion

Our laboratory is focusing on the use of conjugate PPS vaccines, with the hope that such a vaccine will be efficacious against the related diseases, melioidosis and glanders. As discussed, there are additional meritorious approaches to melioidosis immunoprophylaxis. With all of these approaches, however, it is imperative that a standardized animal model be used

for the testing and optimization of immunization regimes. Guinea-pigs, Syrian golden hamsters and certain mice varieties have demonstrated susceptibility to melioidosis and may therefore be suitable animal models [41,52]. However, goats, sheep and to a moderate extent pigs, are livestock animals that are susceptible to melioidosis in endemic areas [53]. These animals may therefore be more appropriate for vaccine testing, as they may be recipients of a finalized vaccine.

Five-year view

Currently, no licensed vaccine exists for prevention of melioidosis or glanders. Within 5 years, it is anticipated that vaccine development in animal models will have been much further investigated, including the use of a conjugate PPS in the study of immunoprophylaxis for both melioidosis and glanders. Trials of this vaccine in horses for protection against glanders will have been completed and the vaccine may become licensed for use in humans for protection against glanders. If this situation arises, the efficacy of this vaccine for protection against the related disease melioidosis may have been initiated in human trials in endemic areas. Therefore, it would appear feasible that the first human melioidosis vaccine could see use within 5 years, although licensing of such a vaccine for the specific purpose of melioidosis immunoprophylaxis could take substantially longer.

In addition, 5 years should allow for a much greater understanding of the molecular actions of *B. pseudomallei* in the host system. This is an area of research that is moving quickly, especially with the advent of the completed *B. pseudomallei* genome sequence, which will be used extensively for genome mining purposes. A better understanding of *B. pseudomallei* pathogenesis can only serve to broaden our perspectives on combating this pathogen, which includes the potential for development of additional vaccination strategies.

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Key issues

- Testing of a protein-polysaccharide conjugate vaccine against glanders in the horse model with the potential to draw parallels to the closely related melioidosis disease in humans.
- Numerous strategies for vaccine development, including conjugate, DNA, attenuated and heterologous vaccines, have all demonstrated potential for further development.
- Studying the underlying mechanisms of *Burkholderia pseudomallei* pathogenesis will help to identify new potential targets for immunoprophylaxis.

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•• of considerable interest

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BIOGRAPHICAL SKETCH

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RESEARCH SUPPORT

Melioidosis Pathogenesis

P.I.: Donald E. Woods

Agency: National Centres of Excellence

Period and Funding Level: April, 1998 to March, 2005, \$94,000 per year

Genetic Analysis of *Burkholderia* spp.

P.I.: Donald E. Woods

Agency: Canadian Institutes of Health Research

Period and Funding Level: October, 2002 to September, 2005, \$110,000 per year

Glanders Vaccine Development

P.I.: Donald E. Woods

Agency: Department of Defense, USA

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the ATCC numbers for the *B. mallei* strains are identical in both studies. Until this situation is clarified, it is unlikely that the observations from the use of *C. elegans* as an animal model to study melioidosis and glanders can be given much weight. An additional point regarding the studies of Gan *et al.* [23] is that these authors believe that a soluble toxin might be responsible for the death of *C. elegans*. There is no clinical evidence either in melioidosis or glanders that a toxin is important in the pathogenesis of these diseases.

Concluding remarks

Considerable advances in understanding the diseases caused by *B. pseudomallei* and *B. mallei* have been made using a combination of tools including genetic techniques and animal infection models. The development of animal models allows us to assess the role of several putative virulence determinants in the pathogenesis of disease, and such models will certainly be valuable in ongoing studies.

Adaptation to host environments is based on pathogenic microorganisms evolving complex gene circuits that allow the bacteria to perceive and respond to different growth conditions. Thus, organisms can turn 'on' one set of genes encoding products that let them survive within the host, while turning 'off' a different set of genes required for their survival in the soil. Efforts are now focused on the identification and characterization of genes involved in this adaptive response in *Burkholderia* spp. as a potential new pool of novel targets, and the use of promoter analysis, microarray technology, proteomics and animal infection models will allow investigators to test the hypothesis that the products encoded by

these genes will be important targets for new vaccines and/or therapeutics.

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The use of animal infection models to study the pathogenesis of melioidosis and glanders

Response from Tan and Gan

We agree with Woods that *Burkholderia pseudomallei* and *Burkholderia mallei* should be recognized as important health concerns. Both *Burkholderia* spp. can cause significant endemic disease (melioidosis and glanders) and are potential agents of bioterrorism. The life cycles of these pathogens are

remarkably versatile and adaptive, which poses significant challenges for their treatment and diagnosis. Understanding how these microorganisms cause human disease is an outstanding medical question that will undoubtedly require the concerted effort of many different experimental approaches.

In his article, Woods claims that two recent studies using *Caenorhabditis elegans* as a model host for *B. pseudomallei* infection have reported 'very different' results [1,2], and thus the results from these studies, as they relate to the pathogenesis of melioidosis in higher organisms, consequently cannot be given



Original article

Characterization of experimental equine glanders

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Abstract

Considerable advances in understanding of the disease caused by *Burkholderia mallei* have been made employing a combination of tools including genetic techniques and animal infection models. The development of small animal models has allowed us to assess the role of a number of putative virulence determinants in the pathogenesis of disease due to *B. mallei*. Due to the difficulties in performing active immunization studies in small animals, and due to the fact that the horse is the target mammalian species for glanders, we have initiated experimental studies on glanders in horses. Intratracheal deposition of *B. mallei* produced clinical glanders with organisms being recovered from tissues of infected horses. The model should prove to be of considerable value in our ongoing studies on the pathogenesis and vaccine development for glanders.

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Keywords: Glanders; Equine; Infection

1. Introduction

Glanders is a zoonotic disease, and those individuals whose occupations put them into close contact with infected animals may contract the disease. *Burkholderia mallei* is a host-adapted pathogen that does not persist in nature outside of its equine host [1]. There have been no naturally occurring cases of glanders in North America in the past 60 years, but laboratory workers are still at risk of infection with *B. mallei* via cutaneous [2] and inhalational [3] routes. Human glanders has been described as a painful and loathsome disease from which few recover without antibiotic intervention [4,5]. There is little known about the virulence factors of this organism, but a recent report indicates that the capsular polysaccharide is essential for virulence in hamsters and mice [6]. At present, no effective vaccines are available against this organism, and information on the effective treatment of infections due to this organism with antibiotic therapy is sparse.

Since *B. mallei* is of significance as an agent of bioterrorism (category B, Centers for Disease Control, US) and biological warfare, the development of effective vaccines and treatments is of particular concern. Our understanding of disease caused by *B. mallei* is rapidly emerging, and we are moving forward with our studies on the pathogenesis of glanders in order to develop new and effective vaccines and/or therapies against *B. mallei*. Development of vaccines and treatments can, therefore, provide important items to assist the World Health Organization, to assist in providing a state of preparedness in case of bioterrorism attacks and to assist signatories of the Biological and Toxin Weapons Convention under Article X of the Convention.

The use of animal infection models is central to the study of microbial pathogenesis. In combination with genetic, immunologic and antigen purification techniques, much can be learned regarding the pathogenesis of disease due to microbes. As the long-term objective of our research is to define at a molecular level the pathogenesis of disease due to *B. mallei*, we have utilized a number of models of *B. mallei* infection in our studies.

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The detection of virulence genes in bacteria is of great biologic interest. We developed a method combining PCR-based subtractive hybridization, insertional mutagenesis and an animal infection model for efficiently detecting such virulence genes, and we have applied the method to the pathogen *B. mallei*. Virulence genes specific to the pathogen *B. mallei* were obtained by subtractive hybridization against the related nonpathogenic species, *B. thailandensis*. From these studies, we determined that a polysaccharide capsule is important for *B. mallei* virulence in a hamster infection model for glanders [6,7].

Strains of mice have also been used as models of glanders, and information obtained from these studies has provided additional understanding of the pathophysiology of this disease [8]. A novel approach to studying the pathogenesis of *Burkholderia* spp. by using an invertebrate model, *Caenorhabditis elegans*, was developed by Jeddalo and colleagues [9]. The model unfortunately appears to show little correlation with virulence in vertebrates, as *B. thailandensis*, which is avirulent for humans, kills *C. elegans* readily. Recently, Gan et al. [10] have reported on the use of *C. elegans* for the study of the pathogenesis of *B. mallei*.

Considerable advances in understanding of the disease caused by *B. mallei* have been made employing a combination of tools including genetic techniques and animal infection models. The development of small animal models has allowed us to assess the role of a number of putative virulence determinants in the pathogenesis of disease due to *B. mallei*. Due to the difficulties in performing active immunization studies in small animals, and due to the fact that the horse is the target mammalian species for glanders, we have initiated experimental studies on glanders in horses. In the studies reported herein, we describe our model of experimental glanders in horses, which should prove to be of considerable value in our ongoing studies on pathogenesis and vaccine development for glanders.

2. Materials and methods

2.1. Organisms

B. mallei ATCC 23344 and a mutant of this organism, *B. mallei* DD3008, that does not express the polysaccharide capsule shown to be essential for virulence [6] were grown at 37 °C on LB agar or in LB broth containing 4% glycerol. *B. mallei* ATCC 23344 was serially passaged three times in Syrian hamsters, and stock cultures were maintained at –70 °C by mixing an equal volume of broth culture and 40% glycerol. All experiments were conducted using animal-passaged *B. mallei* ATCC 23344 and *B. mallei* DD3008 with limited laboratory subculture. Brain heart infusion (20 ml) overnight shaking (150 rpm) cultures of *B. mallei* organisms were washed once with phosphate-buffered saline (PBS, pH 7.2) and resuspended in 5 ml PBS to a concentration of 1×10^{10} organisms/ml. Organisms were maintained in biosafety level 3 containment.

2.2. Animals

Animal studies were approved by the Institutional Animal Care Committee and were performed according to the guidelines of the Canadian Council of Animal Care. Six miniature horses were used in these experiments. Animals ranged in weight from 180 to 220 kg, and were between 4 and 8 years old. Four of the horses were male, and two were female. Animals were housed in biosafety level 3 containment in the National Centre for Foreign Animal Disease in Winnipeg, Manitoba, and all experiments were performed in biosafety level 3 containment. Prior to the beginning of experimentation, animals were allowed to acclimatize to their surroundings for a 2-week period. During this time and for the duration of the experiments, they were provided food (one scoop grain mix, one scoop complete pelleted feed and eight scoops of alfalfa cubes) and water ad libitum.

To initiate the infection, each horse was tranquilized and anesthetized. Prior to inoculation, breathing rates ranged from 12 to 24 breaths per minute (brpm). On day 0, a tranquilizing drug was administered IV in the jugular (1.5 ml of Rompun 100 mg/ml). Five minutes later, 3.0 ml of ketamine 100 mg/ml was given IV in the jugular as well. Following smooth sedation, the following procedures were done: clipped mane/tail with scissors, nasal swabs taken, blood samples taken from the jugular vein (10 ml blood for culture, 10 ml of whole blood for serum), clipped entire neck region with Oster A5 clippers and a #40 blade. A surgical prep with hibitane solution and 70% isopropyl alcohol was performed at the site. A 14-g needle was inserted through the cricothyroid cartilage for the intratracheal inoculation; an 8"-16-g catheter was fed into the trachea to the bifurcation of the main bronchi, and 4 ml of inoculum was administered. Temperature was taken when sedated. Recovery was excellent and took 20 min. Five horses (three males, two females) were anesthetized and inoculated intratracheally with 4 ml of a suspension containing 1×10^{10} *B. mallei* ATCC 23344/ml. One horse (male) was anesthetized and inoculated intratracheally with a 4 ml of a suspension containing 1×10^{10} *B. mallei* strain DD3008/ml. Following inoculation, animals were allowed to recover from the anesthesia and maintained on a normal diet of food and water.

Animals were monitored over a 7-day period. Clinical appearance and rectal temperatures were monitored daily, and 10-ml blood samples were taken daily from the jugular vein of each horse and cultured for the presence of bacteria. Daily nasal swabs were obtained to determine the presence of *B. mallei*. Seven days following inoculation, horses were sacrificed and necropsies performed. Samples were taken for bacteriological and histopathological examination.

2.3. Histology

Tissues were prepared for histologic examination by placing them into 10% buffered formalin, pH 7.2. Following a 3-week fixation period, tissues were dehydrated in graded alcohols, embedded in paraffin and cut into sections 6-µm

thick. Mounted sections were stained for light microscopy with hematoxylin and eosin. Samples collected for histopathology included muzzle, nostril, nasal septa, mandibular lymph nodes, thyroid, mediastinal lymph nodes, lungs, heart, aorta, esophagus, tracheal rings, thymus, salivary glands, liver, spleen, adrenals, pancreas, mesenteric lymph nodes, stomach, duodenum, jejunum, ileum, cecum, proximal colon, distal colon, urinary bladder, mammary gland or prepuce and testicles, brain, pituitary, tongue, soft palate, larynx, haired skin, skeletal muscle and femoral bone marrow.

2.4. Bacteriology

Approximately 5 g of tissue were placed in 3 ml PBS in conical tubes. The tissues were homogenized with a Brinkman Polytron Homogenizer. Homogenates in PBS were plated on four different media including BHI agar (Difco) containing 5% sheep blood and 4% glycerol, Columbia CNA Agar (Difco) containing 5% sheep blood, a selective trypticase soy-based agar containing 1% glycerol, 1000 units polymyxin E, 1250 units bacitracin and 0.25 mg actidione per 100 ml [11] and MacConkey Agar (Difco). Agar plates were streaked on four quadrants to achieve progressive dilution of the inoculum and to assess the relative number of colony-forming units of each microorganism recovered after incubation at 37 °C for up to 96 h. The Biolog system (Hayward, CA) and traditional biochemical tests [12] were used to identify bacterial isolates. Results were expressed as *B. mallei* positive or negative for each tissue. Daily blood cultures and nasal cultures were reported as positive or negative for the presence of *B. mallei*. Samples collected for bacteriology included lung, spleen, liver, kidney, nasal swabs, nasal mucosa, mandibular lymph node, mediastinal lymph node, larynx, laryngotracheal swab, trachea, brain, salivary gland and blood.

3. Results

3.1. Clinical course

The clinical course for all five horses inoculated with *B. mallei* ATCC 23344 was virtually identical. The clinical course for the horse inoculated with *B. mallei* DD3008 (horse 3) was one of a normal, healthy animal throughout the duration of these studies.

On day 1, the rectal temperatures for all of the *B. mallei* ATCC 23344-infected horses had increased to >40 °C except horse 1 which took 2 days to attain this temperature; respiratory rates averaged 24 brpm. Infected animals were showing signs of mild depression, decreased food uptake and defecation. Rectal temperatures for all of the *B. mallei* ATCC 23344-infected horses remained at >39 °C for the remaining time of the studies, while the rectal temperature of the animal infected with *B. mallei* DD3008 remained between 36 and 37 °C for the 7-day period (Fig. 1). On day 2, respiratory rate had decreased to an average of 12 brpm. *B. mallei* ATCC

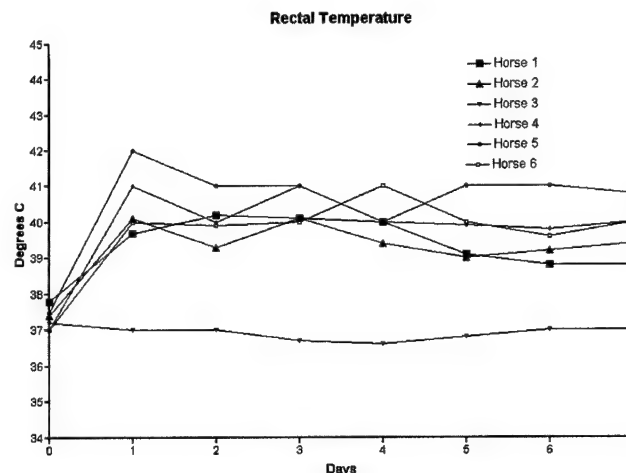


Fig. 1. Rectal temperatures on seven horses over a 7-day period. Horses 1, 2, 4, 5 and 6 were intratracheally inoculated with *B. mallei* ATCC 23344. Horse 3 was intratracheally inoculated with *B. mallei* DD3008 (capsule-minus mutant).

23344-infected horses continued to be mildly depressed with decreased appetite and fecal volume. Horse vocalization was harsh, softer than normal and moist sounding. On day 3, a moderate amount of purulent nasal discharge from the nostrils was noted. On day 4, abdominal lift when breathing was noted. Mild depression and decreased appetite continued. Nasal discharge and crusting of the nostrils were noted. On day 5, bilateral purulent nasal discharge and crusting was noted (Fig. 2). Significant depression and decreased fecal volume continued. On day 6, horses were depressed with significantly decreased appetite and fecal volume. Purulent nasal discharges were noted in all horses that received *B. mallei* ATCC 23344. Clumps of mucus were present all over the sides of the horses and on walls and floors of the cubicles. Abdominal lift when breathing was noted. Bloody crusting was found on the nostril of several of the horses. Bilateral purulent ocular discharge was noted (Fig. 3). Horses ap-

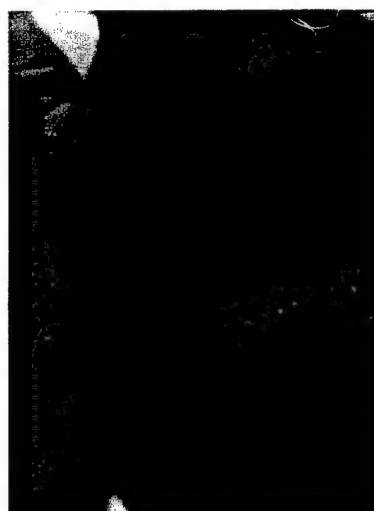


Fig. 2. Photograph of horse intratracheally inoculated with *B. mallei* ATCC 23344 7 days post-inoculation demonstrating bilateral purulent nasal discharge.

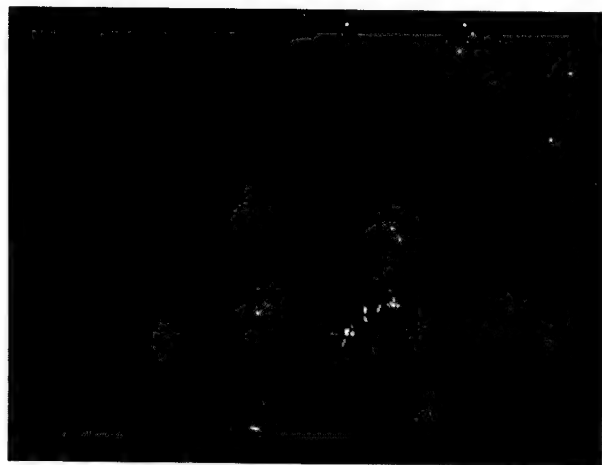


Fig. 3. Photograph of horse intratracheally inoculated with *B. mallei* ATCC 23344 7 days post-inoculation demonstrating ocular discharge.

peared to be cross-stepping on hind ends, and hind legs were spread apart diagonally (Fig. 4). On day 7, breathing was labored with abdominal lift breathing. Bilateral purulent nasal and ocular discharge and crusting was noted with blood-encrusted material on several nostrils. Feces were moist and clumped. Appetite was decreased.

At this point, the decision was made to euthanize the horses. Horses were euthanized by first tranquilizing and anesthetizing the animal. A tranquilization drug was administered IV in the jugular (1.5 ml of Rompun 100 mg/ml). Five minutes later, 3.0 ml of ketamine 100 mg/ml was given IV in the jugular as well. Approximately 500 ml of whole blood and 20 ml whole blood in heparinized tubes were obtained per horse.

3.2. Necropsy results

The following is a representation of the findings on necropsy illustrating the gross pathological findings in the horses inoculated with *B. mallei*-ATCC 23344.

The animals were in good nutritional condition and in a very good state of preservation. The coats were well groomed.



Fig. 4. Photograph of horse intratracheally inoculated with *B. mallei* ATCC 23344 7 days post-inoculation demonstrating diagonally spread hind legs.



Fig. 5. Photograph of excised trachea taken from horse intratracheally inoculated with *B. mallei* ATCC 23344 demonstrating massive amounts of frothy material indicative of pulmonary edema.

The bodies were in rigor mortis. There was bloody material in the nostrils. The eyes were thinly encrusted with dried yellow-gray mucoid material. There were fresh venipuncture sites in the left jugular furrow. In the mucosal lining of the nasal septa, there were multiple ulcerated and deeply reddened areas. The mandibular lymph nodes were moderately enlarged, reddened, wet and bulging on cut surface. Within the caudal larynx, there was a faint mucosal defect (possibly the site of the percutaneous inoculation site.) The trachea was filled with blood-tinged froth (Fig. 5). All lung lobes were diffusely wet, heavy, noncollapsed, dark tan-pink, with rib impressions and having scattered irregular linear, often chevron-shaped, red streaks or stripes (Fig. 6). The cervical and mediastinal lymph nodes were moderately enlarged, reddened and bulging on cut surface. There was marked splenomegaly. The stomach was nearly empty of ingesta. The small and large intestines were well filled with moist digesta. The urinary bladder was mildly distended with clear medium-yellow urine. Other organs were unremarkable.

To summarize the necropsy findings: bodies as a whole were in good nutritional condition and fair states of hydra-



Fig. 6. Photograph of excised lungs taken from horse intratracheally inoculated with *B. mallei* ATCC 23344 demonstrating focal areas of necrosis and frothy material indicative of pulmonary edema.

Table 1
Post-mortem culture results from horses inoculated with *Burkholderia mallei*^a

Culture site	Horse 1	Horse 2	Horse 3	Horse 4	Horse 5	Horse 6
Larynx	–	–	–	+	+	+
Trachea	–	–	–	+	–	+
L apical lobe	–	–	–	+	–	–
L middle lobe	–	–	–	–	–	–
R apical lobe	–	–	–	–	–	–
R middle lobe	–	–	–	–	–	–
R diaphragmatic lobe	–	–	–	–	–	–
L diaphragmatic lobe	–	–	–	–	–	–
L nasal mucosa	+	+	–	+	+	+
R nasal mucosa	+	+	–	+	+	+
Mandibular lymph node	–	–	–	–	–	–
Mediastinal lymph node	–	–	–	–	–	–
Spleen	–	–	–	–	–	–
(L) kidney	–	–	–	+	–	–
(R) kidney	–	–	–	–	–	–
Liver	–	–	–	–	–	–
Brain	–	–	–	–	–	–
Salivary gland	–	–	–	+	–	–

^a Horses 1, 2, 4, 5, and 6 were inoculated intratracheally with *B. mallei* ATCC 23344. Horse 3 was inoculated intratracheally with the *B. mallei* mutant derivative DD3008, which does not express the polysaccharide capsule. Horses were euthanized 7 days post-inoculation.

tion. Unilateral epistaxis was seen in several of the horses with bilateral ulcerative and hemorrhagic rhinitis uniformly noted. Moderate congestion or lymphadenopathy was noted in the mandibular, cervical and mediastinal lymph nodes. All lung lobes demonstrated severe diffuse pulmonary edema, with scattered linear hemorrhages or foci of congestion or pneumonia. There was marked congestion in spleens. The ulcerative lesions in the nasal septa are the likely source of the epistaxis, and the lesions resemble the acute lesion depicted in an early reference [13]. Epistaxis, without a history of previous work or other apparent cause, was reported in this same reference to be one of the frequent concomitant findings in glanders. The lung lesions observed are manifestations of experimental infection. The nature of the reddened streaks in the lung lobes awaits microscopic evaluation (manuscript in preparation), but small V-shaped spots of pneumonia were reported in an early gross description of acute glanders. The severe pulmonary edema was interpreted to be life-threatening. Life expectancy was judged likely to have been less than 12 h.

3.3. Bacteriology

Daily nasal swab cultures were performed on all horses. Nasal cultures for all five *B. mallei* ATCC 23344-inoculated horses were positive on day 7, but not before this. One positive blood culture on horse 2 was noted on day 3; however, no additional blood cultures were positive. Table 1 shows post-mortem culture results on tissues from all five *B. mallei* ATCC 23344-inoculated animals (horses 1, 2, 4, 5, 6) and the *B. mallei* DD3008-inoculated horse (horse 3). Cultures of nasal mucosa were consistently positive for *B. mallei* ATCC 23344-inoculated animals. Cultures of the larynx from three of these horses yielded positive cultures for *B. mallei*. The trachea from two of these horses yielded

positive cultures for *B. mallei*. Cultures of internal organs were consistently negative for *B. mallei* with the exception of horse 4 which had *B. mallei* ATCC 23344 growing in the left apical lobe of the lungs, the left kidney and the salivary gland.

A number of opportunistic secondary invaders were isolated from some of the tissues of the *B. mallei* ATCC 23344-inoculated horses, including *Streptococcus zooepidemicus*, *Pasteurella pneumotropica*, *Pasteurella caballi* and *Mannheimia haemolytica*. *S. zooepidemicus*, in small numbers, was isolated from the brain of all *B. mallei* ATCC 23344-inoculated horses, and horse 4 yielded small numbers of *M. haemolytica* and *P. pneumotropica* from this site as well. Horse 3, inoculated with the capsule-minus mutant *B. mallei* DD3008 yielded *S. zooepidemicus* and *P. pneumotropica* from the nasal mucosa only.

4. Discussion

Glanders is a bacterial disease of perissodactyls (odd-toed ungulates) with zoonotic potential that has been known since ancient times [14]. The incubation period is from a few days to many months [15]. Glanders is transmissible to humans by direct contact with sick animals or infected materials. In the untreated acute disease, there is 95% mortality within 3 weeks. A chronic form with abscessation also occurs. While the clinical course of glanders in humans and animals has been well described [2,5,16], experimental equine glanders has not been studied for over 100 years. In the early 1800s, Rayer transmitted glanders from a patient to horses or donkeys systematically with secretions from acute and chronic cases of glanders [17]. In 1904, McFadyean [18] reported on his studies of experimental equine glanders. He used potato cultures of glanders bacilli administered by ingestion to induce glanders in four horses.

Due to the nonquantitative nature of the above-described early attempts to induce experimental glanders in horses, these experiments would be difficult to repeat. In the present studies, we have described a reproducible model of experimental glanders in the horse. Intratracheal deposition of specific number of organisms has resulted in the development of clinical glanders in the horse, and the model will permit the use of the horse to define the pathogenesis of glanders. The clinical presentation of glanders described in our studies parallels those of earlier studies [17,18] in that we noted that inflammatory nodules and ulcers developed in the nasal passages and gave rise to a sticky yellow discharge, accompanied by enlarged firm submaxillary lymph nodes. The animals exhibited progressive debility, febrile episodes and dyspnoea.

A finding noted in the present studies but not reported in earlier studies is the apparent neurological degeneration in acute glanders. Some of the horses inoculated with *B. mallei* ATCC 23344 exhibited cross-stepping and splaying of the hind legs. There could be a number of reasons for this, but the fact that small numbers of *S. zooepidemicus* were isolated from the brain of all *B. mallei* ATCC 23344-inoculated horses suggests that the blood–brain barrier for infectious agents was compromised in these animals, and the CNS infection might have contributed to the neurological signs. Although these neurological manifestations have not been noted before in acute glanders, this is well recognized in animals with melioidosis, including horses and is likely to reflect direct invasion of the brainstem/spinal cord [19,20]. The fact that *B. mallei* was not isolated more widely from the tissues is likely due to small sample sizes. The fact that *S. zooepidemicus* was isolated from the brain of all *B. mallei* ATCC 23344-inoculated horses, and not from the capsule-minus mutant *B. mallei* DD3008-inoculated animal, argues against this being post-mortem contamination.

The fact that pulmonary edema was a significant contributor to the mode of death of horses infected with *B. mallei* ATCC 23344 may be explained by the fact that horses are obligate nasal breathers, and all the air needed for gas exchange, at rest and during exercise, must pass through the upper airway [21]. It is well described in horses that airway obstruction is associated with pulmonary edema [22]. The massive amounts of sticky yellow mucus present in the nasal passages of *B. mallei*-infected horses could certainly lead to airway obstruction and subsequent pulmonary edema.

In the present studies, we have provided evidence to indicate that the extracellular polysaccharide capsule present on *B. mallei* may be an important virulence determinant for the production of clinical glanders in the horse. The complete inability of a capsule-minus mutant of *B. mallei* (strain DD3008, [6]) to infect the horse provides strong evidence for the importance of the capsule in the pathogenesis of glanders. We are very excited about the possibilities for the use of the model in further studies to define the pathogenesis of glanders, recognizing that this acute model of disease may not reflect all aspects of natural infection.

Importantly, the equine model of glanders that we have developed will allow us to perform active immunization studies to test vaccine candidates for their ability to protect the horse against *B. mallei* infections. We are currently investigating the use of a protein–polysaccharide (PPS) conjugate vaccine in a horse model of glanders. The conjugate consists of the capsular polysaccharide (CPS) and the *O*-polysaccharide from the lipopolysaccharide (*O*-PS) molecules from *B. mallei* conjugated to exotoxin A of *Pseudomonas aeruginosa*, which is used as a carrier molecule to facilitate an immune response [23]. We have reason to believe that such a conjugate will be efficacious against glanders in horses and may therefore become a glanders vaccine for use in humans.

The CPS and *O*-PS structures (capsule and LPS) of *B. mallei* are immunologically related to those of *B. pseudomallei*, as antibodies generated against the CPS and *O*-PS of one cross-react with the other [6,24]. In addition, structural studies of the *O*-PS from LPS are shown to be almost identical between the two species, except that *B. mallei* LPS lacks an *O*-acetyl group on the talose residue [24]. The genes required for the generation of capsule in *B. pseudomallei* and *B. mallei* were also shown to be virtually identical [6,25], supporting the close relation between the CPS structures of these pathogens. Due to the relatedness of the CPS and *O*-PS molecules between *B. pseudomallei* and *B. mallei*, we predict that this vaccine will also provide protection against melioidosis in humans. If this conjugate becomes licensed for human use against glanders, we could then proceed to human trials in areas endemic for melioidosis.

The development of a reproducible model of equine glanders should prove to be of considerable value in our ongoing studies on the pathogenesis and the development of vaccines for glanders. These studies gain added importance with the understanding that *B. mallei* is of significance as an agent of bioterrorism (category B, Centers for Disease Control, US) and biological warfare. Development of vaccines and treatments can, therefore, provide important items to assist in providing a state of preparedness in case of bioterrorism attacks and to assist signatories of the Biological and Toxin Weapons Convention under Article X of the Convention.

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Molecular and Physical Characterization of *Burkholderia mallei* O Antigens

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***Burkholderia mallei* lipopolysaccharide (LPS) has been previously shown to cross-react with polyclonal antibodies raised against *B. pseudomallei* LPS; however, we observed that *B. mallei* LPS does not react with a monoclonal antibody (Pp-PS-W) specific for *B. pseudomallei* O polysaccharide (O-PS). In this study, we identified the O-PS biosynthetic gene cluster from *B. mallei* ATCC 23344 and subsequently characterized the molecular structure of the O-PS produced by this organism.**

Burkholderia mallei is a gram-negative bacterium responsible for a disease known as glanders in solipeds and occasionally in humans (3, 8, 13). The factors involved in the pathogenesis of *B. mallei* infection remain relatively poorly defined at the molecular level. A previous study that identified a polysaccharide gene cluster in *B. mallei* showed that *B. mallei* lipopolysaccharide (LPS) cross-reacts with polyclonal antibodies raised against the LPS of *Burkholderia pseudomallei*, a closely related organism responsible for a disease known as melioidosis (6). In the present study, we investigated the LPS profiles of *B. mallei* strains, identified the gene cluster responsible for O polysaccharide (O-PS) biosynthesis in *B. mallei* ATCC 23344, and determined the physical structure of the *B. mallei* ATCC 23344 O-PS. Additionally, we showed that the O-PS moiety of *B. mallei* LPS is required for resistance to the bactericidal action of serum. Finally, we identified the presence of insertion sequences in two strains of *B. mallei* that disrupt the expression of O-PS.

Analysis of LPS profiles of *B. mallei* strains. The strains and plasmids used in this study are shown in Table 1. The first goal of this study was to assess the LPS profiles of *B. mallei* strains. Initially, we performed Western blot analysis of *B. mallei* ATCC 23344 whole-cell lysates with polyclonal rabbit sera raised against a *B. pseudomallei* bovine serum albumin (BSA)-O-PS conjugate as well as with a *B. pseudomallei* O-PS-specific MAb (Pp-PS-W) according to a previously described protocol (1, 2). As shown in Fig. 1A, *B. mallei* ATCC 23344 reacted with the anti-LPS polyclonal sera, resulting in a typical LPS banding pattern; however, the *B. pseudomallei* O-PS-specific MAb (Pp-PS-W) did not react. This indicated that differences exist between *B. mallei* and *B. pseudomallei* O-PS. We further assessed the LPS profiles of 10 different *B. mallei* strains (Fig. 1B). By using Western blot analysis, we showed that 8 of the 10 strains assessed bound the anti-LPS polyclonal sera and displayed typical LPS banding patterns. In contrast, however, two strains, NCTC 120 and ATCC 15310, did not bind

the anti-LPS polyclonal sera, as indicated by the absence of bands (Fig. 1B). In order to confirm that the O-PS moiety was absent rather than a different type of O-PS, silver stain analysis was employed. Figure 1C shows the silver stain results confirming that both of these strains lacked O-PS moieties.

Identification and characterization of *B. mallei* ATCC 23344 O-PS biosynthetic gene cluster. In order to investigate the genes responsible for O-PS biosynthesis in *B. mallei*, we constructed a cosmid library by using *B. mallei* ATCC 23344 genomic DNA and the cosmid pScosBC1 by using a previously described protocol (12). Colony hybridizations were then performed with a 1.1-kb DNA fragment containing the recently identified *B. mallei* *wbiA* gene (P. Brett, M. Burtneck, and D. Woods, unpublished data). Six positive cosmid clones were obtained. Based on the *Bam*HI-*Kpn*I restriction patterns obtained, two cosmid clones, 1C3 and 2B5, were predicted to harbor the entire *B. mallei* O-PS gene cluster. Sequence analysis resulted in 19,918 bp of contiguous sequence containing the entire *B. mallei* O-PS biosynthetic gene cluster with an IS407-like insertion sequence element at the 3' end.

The first 18,738 bp of the *B. mallei* DNA sequence contained 16 predicted ORFs that were identical to those previously defined as the O-PS biosynthetic gene cluster in *B. pseudomallei* (Fig. 2) (5). Sequence alignment of the *B. pseudomallei* and *B. mallei* O-PS biosynthetic regions revealed 99% identity at the nucleotide level. The genes comprising the *B. mallei* O-PS biosynthetic operon were named as per the identical genes found in *B. pseudomallei* (5).

Physical characterization of *B. mallei* O-PS moieties. In order to structurally analyze the *B. mallei* O-PS structure, it was necessary to construct a *B. mallei* strain unable to produce capsular polysaccharide (CPS), because CPS copurifies with LPS. The suicide vector pGSV3008 was employed as previously described to construct *B. mallei* PB100, a derivative of ATCC 23344 that does not produce CPS (6). The O-PS was purified as previously described for *B. pseudomallei*. Figure 3 shows ¹³C nuclear magnetic resonance (¹³C-NMR) analysis (Complex Carbohydrate Research Center, University of Georgia, Athens) results demonstrating that the *B. mallei* O-PS

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TABLE 1. Bacterial strains and cosmids or plasmids used in this study

Strain, cosmid, or plasmid	Relevant characteristic(s)	Source or reference
Strains		
<i>E. coli</i>		
SM10	Mobilizing strain: transfer genes of RP4 integrated into the chromosome; Km ^r Sm ^s	11
TOP10	High-efficiency transformation strain with blue/white screening; Ap ^s Km ^s	Invitrogen
HB101	Serum-sensitive strain	7
<i>B. mallei</i>		
NCTC 120		USAMRIID ^a
NCTC 10248	Human isolate	USAMRIID
NCTC 10229		USAMRIID
NCTC 10260	Human isolate	USAMRIID
NCTC 10247		USAMRIID
ATCC 23344	Human isolate; Pm ^r Gm ^s	USAMRIID
NCTC 3708	Mule isolate	USAMRIID
NCTC 3709	Horse isolate	USAMRIID
ATCC 10399		USAMRIID
ATCC 15310		USAMRIID
PB100	ATCC 23344::pGSV3008; Pm ^r Gm ^r	This study
<i>B. pseudomallei</i> 1026b	Clinical isolate; Gm ^r Km ^r Sm ^r Pm ^r Tp ^r	4
Cosmids		
pScosBC1	Broad-host-range cosmid cloning vector based on pSuperCos1; Ap ^r Tp ^r	12
p1C3	pScosBC1 from ATCC 23344 library with a 23-kb fragment containing the O-PS biosynthetic gene cluster	This study
p2B5	pScosBC1 from ATCC 23344 library with a 27-kb fragment containing the O-PS biosynthetic gene cluster	This study
Plasmids		
pUC19	Cloning vector with blue/white selection; Ap ^r	14
pGSV3008	pGSV containing a 379-bp <i>Eco</i> RI fragment from pDD3008, contains internal fragment from the <i>wcbB</i> gene; Gm ^r	6

^a U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Md.

backbone is similar to that previously described for *B. pseudomallei* O-PS, a heteropolymer of repeating D-glucose and L-talose (9, 10). However, changes are apparent in the O-acetylation pattern of the *B. mallei* L-talose residue in comparison to that of *B. pseudomallei*. Similar to *B. pseudomallei* O-PS, *B. mallei* O-PS demonstrates the presence of O-acetyl or O-methyl substitutions at the 2' position of the talose residue. In contrast, *B. mallei* O-PS is devoid of an O-acetyl group at the 4' position of the talose residue. Thus, the structure of *B. mallei* O-PS is best described as 3)-β-D-glucopyranose-(1,3)-6-

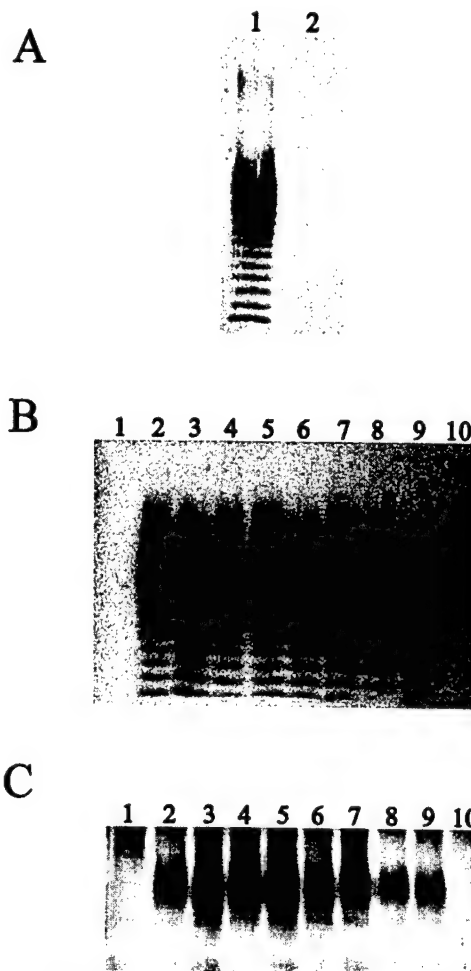
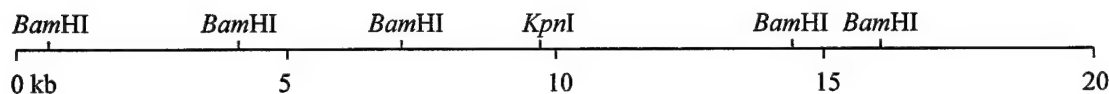


FIG. 1. (A) Western blot analysis of *B. mallei* ATCC 23344. Proteinase K-treated whole-cell lysates were used. In lane 1, the primary antibody used was a 1/2,000 dilution of polyclonal antisera raised against a *B. pseudomallei* BSA-O-PS conjugate, and in lane 2, the primary antibody used was a 1/2,000 dilution of the *B. pseudomallei* O-PS-specific MAb (Pp-PS-W). (B) Western blot profiles of proteinase K-treated whole-cell lysates of *B. mallei* strains. The primary antibody used was polyclonal sera raised against a *B. pseudomallei* BSA-O-PS conjugate. Lanes: 1, NCTC 120; 2, NCTC 10248; 3, NCTC 10229; 4, NCTC 10260; 5, NCTC 10247; 6, ATCC 23344; 7, NCTC 3708; 8, NCTC 3709; 9, ATCC 10399; and 10, ATCC 15310. (C) Silver stain analysis of proteinase K-treated whole-cell lysates of *B. mallei* strains. Lanes: 1, NCTC 120; 2, NCTC 10248; 3, NCTC 10229; 4, NCTC 10260; 5, NCTC 10247; 6, ATCC 23344; 7, NCTC 3708; 8, NCTC 3709; 9, ATCC 10399; and 10, ATCC 15310.

deoxy-α-L-talopyranose-(1-, in which the talose residue contains 2-O-methyl or 2-O-acetyl substituents. Recent studies indicate that the presence of 4-O-acetyl groups on the talose residues of *B. pseudomallei* O-PS is due to an O-acetylation locus unlinked to the previously described O-PS biosynthetic operon (Brett et al., unpublished). If this is the case, then the unlinked locus responsible for O-acetylation is either not present or is nonfunctional in *B. mallei* strains. The presence or absence of O-acetyl groups on the O-PS moieties may have consequences when O-PS is considered as a component of a vaccine that protects against both *B. mallei* and *B. pseudo-*

A



B

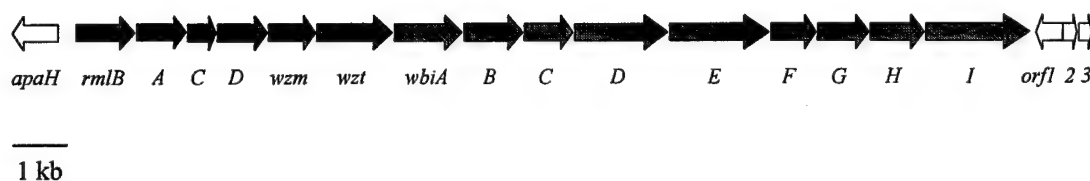


FIG. 2. Restriction and genetic maps of the *B. mallei* O-PS biosynthetic gene cluster. (A) Restriction map. The horizontal line represents the *B. mallei* DNA insert of cosmid 2B5. The locations of *Bam*HI and *Kpn*I cleavage sites used for subcloning are shown. Two additional *Bam*HI sites at the 5' and 3' ends of 2B5, which were part of the pScosBC1 vector, are not shown. (B) Genetic map. The location and direction of transcription of the genes are represented by arrows, and the gene names are shown below. The gray arrows indicate the genes involved in O-PS biosynthesis based on homology to the *B. pseudomallei* O-PS biosynthetic operon.

***B. mallei* survives in 30% NHS, and serum-sensitive strains lack the O-PS moiety of LPS.** The ability of *B. mallei* ATCC 23344 to grow in the presence of 30% normal human serum (NHS) was initially assessed with a serum bactericidal assay (5) in which viable counts were determined at 2, 4, 8, and 18 h. *B. mallei* ATCC 23344 was shown to survive in the presence of 30% NHS over the course of 18 h (Fig. 4A). Serum-resistant *B. pseudomallei* 1026b and serum-sensitive *Escherichia coli* HB101 were employed as controls.

In order to assess the role of *B. mallei* O-PS in serum resistance, NHS bactericidal assays (5) were performed with *B. mallei* ATCC 23344 and *B. mallei* NCTC 120 and ATCC 15310, the two strains lacking O-PS. *B. mallei* ATCC 23344 remained resistant to the killing action of 30% NHS, while NCTC 120 and ATCC 15310 were completely killed following a 2-h incubation in 30% NHS (Fig. 4B). The other seven *B. mallei* strains used in this study possessed intact O-PS moieties and were resistant to the bactericidal action of 30% NHS (data not shown). These results suggested that *B. mallei* O-PS moieties play a crucial role in the serum resistance of this organism; this correlates well with previous studies demonstrating that *B. pseudomallei* O-PS is required for serum resistance (5).

Identification of insertion sequence IS407 in the O-PS biosynthetic gene clusters of *B. mallei* NCTC 120 and ATCC 15310. In order to determine if the O-PS biosynthetic gene clusters of NCTC 120 and ATCC 15310 had been disrupted, we chose to individually PCR amplify each gene present in this cluster. Deoxyoligonucleotide primers were designed outside of the 5' and 3' ends of each gene. *B. mallei* ATCC 23344

chromosomal DNA was used as a control as an indicator of the size of a wild-type copy of each gene. Alterations were observed in the *wbiE* PCR product from NCTC 120 and in the *wbiG* PCR product from ATCC 15310. The PCR products obtained in both cases were approximately 1.5 kb larger than those obtained with ATCC 23344 genomic DNA (data not shown). Cloning and sequence analysis of the NCTC 120 *wbiE* and ATCC 15310 *wbiG* PCR products revealed the presence of insertion sequences within these two genes. In NCTC 120, an IS407-like element was located after nucleotide 13615 of the O-PS operon in the *wbiE* gene. In ATCC 15310, an IS407-like element was located following nucleotide 15107 of the O-PS operon in the *wbiG* gene. It is likely that the presence of insertion elements in the O-PS biosynthetic gene clusters of *B. mallei* NCTC 120 and ATCC 15310 is responsible for the loss of expression of O-PS in these two strains. DeShazer et al. have previously demonstrated the presence of an IS407-like element (termed "IS407A") at the 3' end of the CPS gene cluster and have shown that this element is active in *B. mallei* (6). The data presented in this paper certainly support the view that IS407 is functionally active in *B. mallei*.

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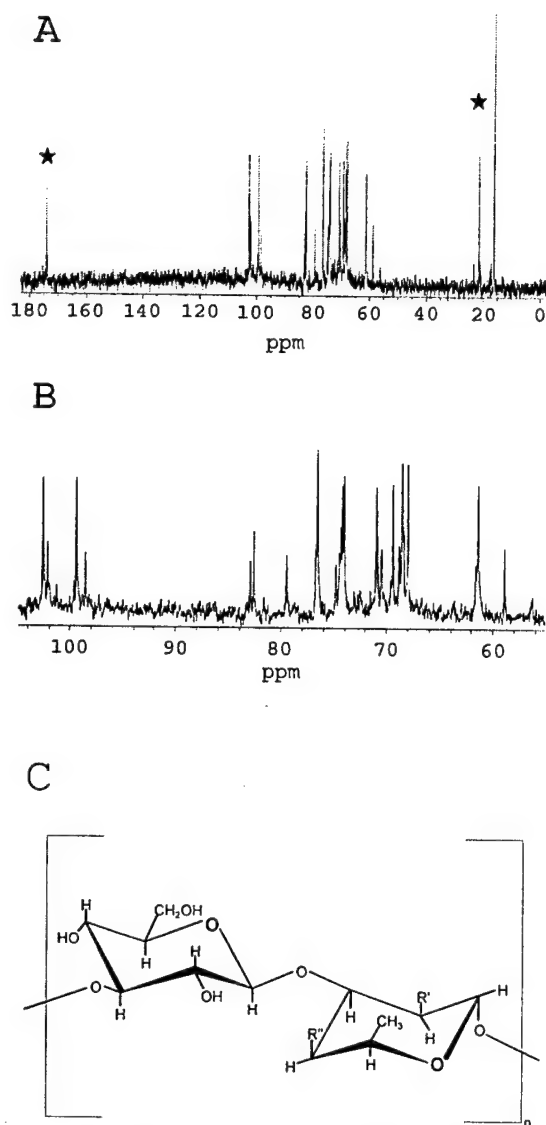


FIG. 3. ¹³C-NMR analysis of *B. mallei* PB100 O-PS. (A) O-Acetyl peaks are indicated by stars. (B) Expanded view of the region running from 100 to 60 ppm. (C) Structure of *B. pseudomallei* and *B. mallei* O-PS. In *B. pseudomallei*, in 33% of the talose residues, R' = O-methyl and R'' = O-acetyl, and in 66% of the talose residues, R' = O-acetyl and R'' = OH. In *B. mallei*, R' = O-acetyl or O-methyl and R'' = OH.

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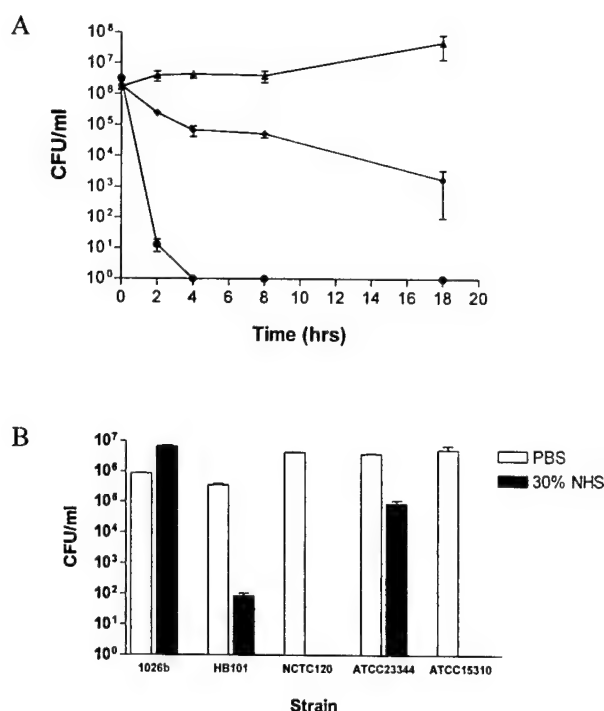


FIG. 4. Serum bactericidal assays with *B. mallei* strains. (A) Thirty percent NHS killing assay in which viable counts were determined at the 2-, 4-, 8-, and 18-h time points. *B. pseudomallei* 1026b (▲), *B. mallei* ATCC 23344 (◆), and *E. coli* HB101 (●). (B) Thirty percent NHS killing assay in which viable counts were determined following a 2-h incubation at 37°C. Control tubes containing phosphate-buffered saline (PBS) are shown as white bars, and experimental tubes containing 30% NHS are shown as gray bars. Error bars indicate standard deviations.

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The *wbiA* locus is required for the 2-*O*-acetylation of lipopolysaccharides expressed by *Burkholderia pseudomallei* and *Burkholderia thailandensis*

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Abstract

Burkholderia pseudomallei and *Burkholderia thailandensis* express similar O-antigens (O-PS II) in which their 6-deoxy- α -L-talopyranosyl (L-6dTalp) residues are variably substituted with *O*-acetyl groups at the *O*-2 or *O*-4 positions. In previous studies we demonstrated that the protective monoclonal antibody, Pp-PS-W, reacted with O-PS II expressed by wild-type *B. pseudomallei* strains but not by a *B. pseudomallei wbiA* null mutant. In the present study we demonstrate that WbiA activity is required for the acetylation of the L-6dTalp residues at the *O*-2 position and that structural modification of O-PS II molecules at this site is critical for recognition by Pp-PS-W. © 2002 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: *Burkholderia* species; O-antigen; Virulence determinant; *trans*-Acylase

1. Introduction

Burkholderia pseudomallei, the etiologic agent of melioidosis, is a Gram-negative bacterial pathogen responsible for disease in both humans and animals [1,2]. Previous studies have demonstrated that the lipopolysaccharide (LPS) expressed by *B. pseudomallei* is both a virulence determinant and a protective antigen [3–6]. Consequently, the O-antigen (O-PS II) has become a significant component of the various sub-unit vaccine candidates that we are currently developing for immunization against melioidosis [7].

The O-PS II moiety produced by *B. pseudomallei* is an unbranched heteropolymer consisting of disaccharide repeats having the structure 3)- β -D-glucopyranose-(1 \rightarrow 3)-6-deoxy- α -L-talopyranose-(1 \rightarrow in which ~33% of the 6-deoxy- α -L-talopyranose (L-6dTalp) residues possess 2-*O*-methyl and 4-*O*-acetyl substitutions while the remainder of the L-6dTalp residues bear only 2-*O*-acetyl modifica-

tions [8,9]. Studies have also demonstrated that the non-pathogenic species *Burkholderia thailandensis* synthesizes an O-antigen with the same repeating unit [10]. Recently, we demonstrated that the O-antigen (O-PS) expressed by *Burkholderia mallei*, the causative agent of glanders, is virtually identical to O-PS II except that it lacks acetyl modifications at the *O*-4 position of the L-6dTalp residues [11]. Curiously, however, pairwise comparisons between the *B. mallei* and *B. pseudomallei* O-polysaccharide biosynthetic clusters failed to reveal any sequence differences that could account for the structural dissimilarities observed between O-PS and O-PS II [5,11].

In the current study, we used a combination of molecular and physical approaches to further characterize the role of the *wbiA* locus which is thought to be involved in the acetylation of O-PS II antigens [5].

2. Materials and methods

2.1. Strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are described in Table 1. *Escherichia coli*, *B. pseudomallei* and *B. thailandensis* strains were grown at 37°C in Luria–Bertani (LB) broth or on LB agar. *B. mallei* strains were

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grown at 37°C in LB broth containing 4% glycerol or on LB agar containing 4% glycerol. For *E. coli*, antibiotics were used at the following concentrations: ampicillin (Ap) 100 µg ml⁻¹, gentamicin (Gm) 15 µg ml⁻¹ and kanamycin (Km) 25 µg ml⁻¹. For *B. pseudomallei* and *B. thailandensis*, antibiotics were used at the following concentrations: Gm 25 µg ml⁻¹, streptomycin (Sm) 100 µg ml⁻¹ and trimethoprim (Tp) 100 µg ml⁻¹. Bacterial strains were maintained at -70°C in 20% glycerol suspensions.

2.2. DNA manipulations and transformations

Molecular and cloning techniques were performed essentially as described by Sambrook et al. [12]. Plasmids were purified using QIAprep spin plasmid minipreps (Qiagen). Genomic DNA was isolated using the Wizard[®] Genomic DNA Isolation kit (Promega). Competent *E. coli* were transformed using standard methods.

2.3. PCR amplification and sequence analysis of *whiA* genes

The *whiA* genes from *B. thailandensis* ATCC 700388 and *B. pseudomallei* 1026b were PCR amplified from purified chromosomal DNA samples using the *whiA*-5' (5'-GCTCTAGACATGAGATCGTGCTTGAGCG-3') and *whiA*-3' (5'-GGGGTACCGATAAAGCCAGCCCCACCGG-3') primer pair; the *Xba*I and *Kpn*I sites in the linker regions are underlined. The primers were designed at the 3'-end of *wzt* and the 5'-end of *whiB* using the previously described *B. pseudomallei* O-PS II biosynthetic gene cluster (GenBank database accession number AF064070). Reactions were performed using *Taq* polymerase (Invitrogen) as per manufacturer's instructions except that the denaturing temperature was increased to 97°C to compensate for the high G/C content of the chromosomal DNAs. The resulting PCR products were then cloned into pCR2.1-TOPO and sequenced on both strands. Sequence analyses were conducted with the aid of DNASIS version 2.5 (Hitachi) as well as the BLASTX and BLASTP programs [13]. The *Shigella flexneri* bacteriophage SF6 *oac* GenBank accession number is X56800. The *B. thailandensis* nucleotide sequence reported in this study was entered into the GenBank database under accession number AY028370.

2.4. Construction and complementation of *whiA* mutants

B. pseudomallei PB604, a strain harboring an insertionally inactivated *whiA* gene, was previously constructed by DeShazer et al. [5]. The *whiA* gene of *B. thailandensis* was insertionally inactivated using the allelic exchange vector pPB604Tp resulting in strain BT604. Allelic exchange was performed as previously described [5,14]. Mutants were complemented *in trans* using the broad host range vector pUCP31T harboring a wild-type copy of the *B. pseudomallei whiA* locus. Plasmids were conjugated to *B. pseudomallei* and *B. thailandensis* as previously described [15].

2.5. Western blot and silver stain analysis

Whole cell lysates were prepared as previously described [16] and used in both Western immunoblot and silver stain analyses. Overnight bacterial cultures were pelleted, resuspended in lysis buffer and boiled prior to SDS-PAGE analysis on 12% gels. Immunoblots were performed as previously described [17] using rabbit polyclonal antiserum specific for *B. pseudomallei* O-PS II. Silver stain analyses were performed as previously described [18].

2.6. Purification of LPS and O-PS

LPS was purified using a previously described hot aqueous phenol extraction protocol [7,9]. Delipidation of the LPS molecules was achieved via mild acid hydrolysis (2% acetic acid) followed by size exclusion chromatography (Sephadex G-50) as previously described by Perry et al. [9]. Carbohydrate positive fractions were detected using a phenol-sulfuric acid assay [19]. The purity of the carbohydrate preparations was determined to be >90% in all instances. Protein contamination was determined using bicinchoninic acid assays (Pierce) while nucleic acid contamination was estimated from OD_{260/280} measurements.

2.7. Nuclear magnetic resonance (NMR) spectroscopy analysis

¹³C-NMR spectra were recorded at 100.5 MHz and the chemical shifts were recorded in ppm relative to an internal acetone standard (31.07 ppm [¹³C]; Spectral Data Services, Champaign, IL, USA).

3. Results and discussion

3.1. Comparison of *whiA* alleles from *B. thailandensis* and *B. pseudomallei*

The *whiA* allele from *B. thailandensis* ATCC 700388 was cloned and sequenced as described in Section 2. Analysis of the 1239-bp open reading frame contained within the cloned PCR product demonstrated sequence identities of 93.6% at the nucleotide and 95.0% at the amino acid levels in comparison to the previously characterized *B. pseudomallei* 1026b *whiA* allele (Fig. 1). Based upon these preliminary results we predicted that the function of *WhiA* would be similar in both *B. pseudomallei* and *B. thailandensis*.

Further analysis of the *whiA* gene products expressed by the two *Burkholderia* species demonstrated the presence of conserved amino acid motifs that defines a family of inner membrane *trans*-acylases. The structural and functional significance of these motifs, however, has yet to be determined. The family includes *Salmonella typhimuri*-

Table 1
Bacterial strains and plasmids used in this study

Strains and plasmids	Relevant characteristic(s)	Reference or source
Strains		
<i>E. coli</i>		
SM10	Mobilizing strain: expresses RP4 <i>tra</i> genes; Km ^r Sm ^r	[21]
TOP10	High efficiency transformation	Invitrogen
<i>B. pseudomallei</i>		
1026b	Clinical isolate: Gm ^r Km ^r Sm ^r Pm ^r Tp ^s	[5]
DD503	1026b derivative: $\Delta(amrR-oprA)$ <i>rpsL</i> ; Sm ^r Pm ^r Gm ^s Km ^s Tp ^s	[5]
PB604	DD503 derivative: <i>wbiA::dhfrIIb</i> -p15A oriV; Tp ^r	[5]
PB605	PB604 (pUCP31T); Gm ^r Tp ^r	This study
PB606	PB604 (p31wbiA); Gm ^r Tp ^r	This study
<i>B. thailandensis</i>		
ATCC 700338	Type strain (soil isolate): Gm ^r Km ^r Sm ^r Pm ^r Tp ^s	[10]
DW503	ATCC 700338 derivative: <i>rpsL</i> ; Sm ^r Pm ^r Gm ^s Km ^s Tp ^s	[22]
BT604	DW503 derivative: <i>wbiA::dhfrIIb</i> -p15A oriV; Tp ^r	This study
BT605	BT604 (pUCP31T); Gm ^r Tp ^r	This study
BT606	BT604 (p31wbiA); Gm ^r Tp ^r	This study
<i>B. mallei</i>		
ATCC 23344	Type strain (human isolate)	USAMRIID ^a
Plasmids		
pCR2.1-TOPO	TA cloning vector: ColEI ori; Ap ^r Km ^r	Invitrogen
pUCP31T	Broad host range vector: OriT pRO1600 ori; Gm ^r	[23]
p31wbiA	1.37-kb <i>B. pseudomallei</i> <i>wbiA</i> PCR product cloned into the <i>XbaI/KpnI</i> sites of pUCP31T; Gm ^r	This study

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um OafA, *Shigella flexneri* bacteriophage SF6 Oac, *Rhizobium meliloti* ExoZ and *Legionella pneumophila* Lag1 [20]. Interestingly, all are involved in the acetylation of bacterial polysaccharides [20]. A gapped sequence align-

ment of the WbiA homologues with the Oac *trans*-acylase revealed overall sequence identities of approximately 30% (Fig. 1), a result that is consistent with the family in general.

Bp	1	MKSEIPVTVP	DRFDAADVLG	APQSARVADP	STLANPADRL	STHDNGFGLL	50
Bt	1	MKSEIPVTVP	ERFNTADVLG	GAQPTRVADR	STLANPADRL	STHDNGFGLL	50
Sf	1	-----	-----	-----	-----	MHKSNCFDTA	10
Bp	51	RLLFATMVLW	DHAFPLGGFG	ADPMWRLTLN	QDSMGGICVS	GFFAISGFLI	100
Bt	51	RLLFATMVLW	DHAFPLGGFG	ADPMWRLTLN	QDSMGGICVS	GFFAISGFLI	100
Sf	11	RLVAMMVLW	SHHYALSGQP	EP----YLFQ	FESAGGIAVI	IFFSISGYLI	56
Bp	101	AKSGMRAD-A	LQFAWRRCV*	IFPAYWAVLI	VTALCVGPIL	HYVOAGTLHG	149
Bt	101	AKSGMRAD-A	LQFAWRRCV*	IFPAYWAVLI	VTALGVGPIL	HYVOAGTLHG	149
Sf	57	SKSAIRSDSF	IDFMARRR	IFPALVPCST	LTFLFLGWIL	NDFSAEYFS-	105
Bp	150	YWNALGGPL	GYIYNNWRLT	IGQYGINDLL	RDTTPYGHSI	SESVFNGSIW	199
Bt	150	YWNALGGPL	GYIYNNWRLT	IGQYGINDLL	RDTTPYGRSI	SESVFNGSIW	199
Sf	105	-----	---HDIVRKT	ISSIFMSQAP	DADITS--HL	IHAGINGSIW	140
Bp	200	TLIYEAKCYV	MVGLFAMFGL	LTÄHRRVLLA	VTVVAWFVLA	IQTINPAFSA	249
Bt	200	TLIYEAKCYV	LVGLFAVFGL	LTEHREPVLLA	VTGVSWLVLA	VQTINPAFSA	249
Sf	141	TLPLEFLCYI	ITG-VAVAH	KNGKAFIVIL	LVFVSLSLIG	SVSENRDVME	189
Bp	250	QLLPWAGDRH	LVQYGTIFLI	GSSAAAYSXS	LPI\$DKLGAF	AVVYVILISLF	299
Bt	250	QLVPWAGDRH	LVQYGTIFLI	GSSAAAYSXS	LPI\$DKLGAF	AVVIYVILISLF	299
Sf	190	SIPLWLYP--	--LRGLAFF	GATMAMYKS	WNV\$NVKITV	VSLAMAYAYA	235
Bp	300	KGG----YLL	LGYPAMVYAI	LWLACRLPRW	ARRIGSRNDY	SYGIYVFGFL	345
Bt	300	KGG----YLL	LGYPAMVYAI	LWLACRLPRW	ARRIGSRNDY	SYGIYVFGFL	345
Sf	236	SYGKGIDYTM	TCYILVSFST	IAICTSVG--	DPLVKGRFDY	SYGVYIYAFP	283
Bp	346	VQOVLAYVGA	YKYGFVFYLA	ASVFFTFICA	WFSWHLIEKR	ALALKDWGPG	395
Bt	346	VQOVLAYVGA	YKYGFVFYLA	ASVFFTFICA	WFSWHLIEKR	ALALKDWGPG	395
Sf	284	VQQVINT--	LHMGFYPSML	LSAVTVLFLS	HL\$WNLVEKR	FLTRSSP---	328
Bp	396	QGWKYCLARL	TMKKEGV	412			
Bt	396	QGWKYCLARL	TMKKEGV	412			
Sf	328	-----KL	SLD----	333			

Fig. 1. Amino acid alignment of *B. pseudomallei* 1026b (Bp) *wbiA*, *B. thailandensis* ATCC 700338 (Bt) *wbiA* and *S. flexneri* phage SF6 (Sf) *oac* gene products. Shaded residues represent identity amongst the aligned sequences. Dots indicate dissimilarities between the Bp and Bt proteins. Asterisks indicate residues conserved amongst members of the family of integral membrane proteins involved in the acylation of exported carbohydrates.

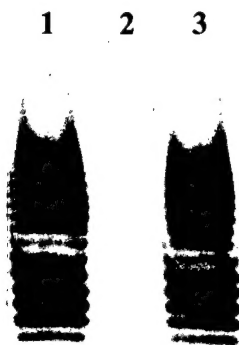


Fig. 2. Western immunoblot analysis of purified *B. thailandensis* LPS antigens. The primary antibody used was the O-PS II specific Pp-PS-W mAb. Lane 1, DW503 LPS; lane 2, BT605 LPS; lane 3, BT606 LPS.

3.2. Phenotypic characterization of *wbiA* null mutants

To determine the effect of the *wbiA* null mutations on the synthesis of O-PS II, *B. pseudomallei* PB604 and *B. thailandensis* BT604 were phenotypically characterized using a variety of genetic and immunological approaches. Silver staining of SDS-PAGE fractionated whole cell lysates demonstrated that BT604 was capable of expressing full-length LPS molecules based upon the presence of a characteristic LPS banding pattern (data not shown). The LPS was also shown to be immunologically similar

to that expressed by the type strain and DW503 due to the reactivity of the antigen with the O-PS II polyclonal antiserum (data not shown). Interestingly, however, neither the BT604 whole cell lysates nor the purified LPS molecules reacted with the O-PS II specific monoclonal antibody (mAb) Pp-PS-W suggesting that the *wbiA* locus was required for the expression of a native O-PS II moieties (Fig. 2). By complementing BT604 with the broad host range vector, p31wbiA, we were able to restore the reactivity of the whole cell lysates and purified LPS with the Pp-PS-W mAb (Fig. 2). Similar results were observed for the *B. pseudomallei* strains (data not shown).

3.3. Spectroscopic analysis of the O-polysaccharide antigens

The O-polysaccharides from *B. thailandensis* DW503, BT604 and BT606, *B. pseudomallei* DD503, PB604 and PB606 and *B. mallei* ATCC 23344 were isolated and purified as described in Section 2. The ^{13}C -NMR spectrum of the DW503 antigen demonstrated four anomeric carbon signals between 98.5 and 102.6 ppm, two O-acetyl signals at 174.1 and 174.6 ppm (CH_3CO) as well as 21.2 and 21.4 ppm (CH_3CO), two 6-deoxyhexose CH_3 signals at 16.0 and 16.2 ppm and an O-methyl signal at 58.8 ppm (Fig. 3A), all of which are consistent with previously published values [9]. Similar spectra were also obtained for the

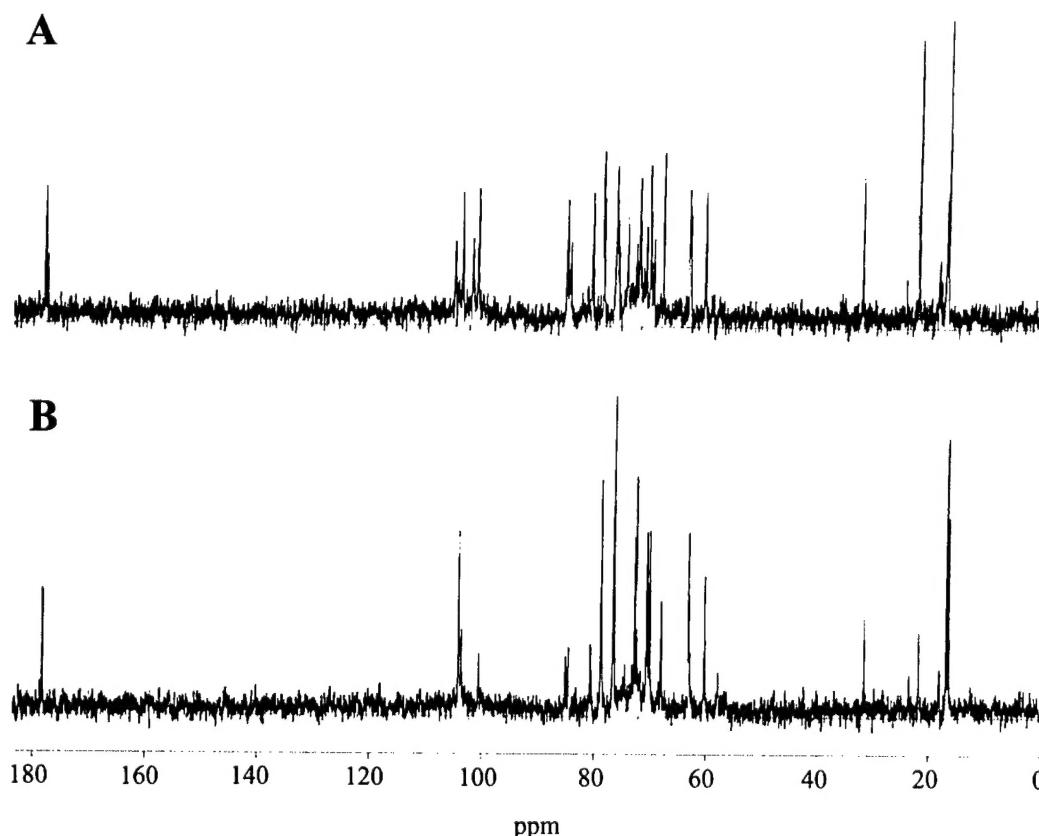


Fig. 3. ^{13}C -NMR spectra of native and mutant O-polysaccharides expressed by *B. thailandensis* strains (A) DW503 and (B) BT604.

BT606, DD503 and PB606 samples (data not shown). In contrast, the ^{13}C -NMR spectrum of the BT604 sample demonstrated four anomeric carbon signals between 98.5 and 102.2 ppm, one *O*-acetyl signal at 174.6 ppm (CH_3CO) and 21.2 (CH_3CO), two 6-deoxyhexose CH_3 signals at 16.0 and 16.3 ppm and an *O*-methyl signal at 58.8 ppm (Fig. 3B). A similar spectrum was recorded for the PB604 sample (data not shown). Based upon these results it was apparent that the O-polysaccharides expressed by BT604 and PB604 were lacking one of the two *O*-acetyl moieties associated with native O-PS II molecules.

To determine which of the *O*-acetyl groups was missing a comparison of the DW503 and BT604 ^{13}C -NMR spectra with the ^{13}C -NMR spectrum obtained for *B. mallei* ATCC 23344 O-PS was conducted. Based upon an analysis of the spectral data we were able to establish that BT604 lacks *O*-acetyl modifications at the *O*-2 position of the L-6dTalp residues since O-polysaccharides lacking *O*-acetyl substitutions only at the *O*-4 position would have produced spec-

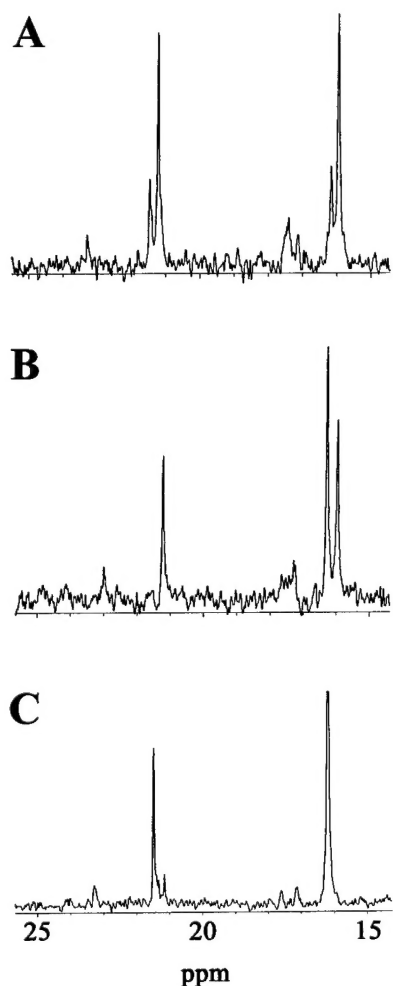


Fig. 4. ^{13}C -NMR spectra of *B. thailandensis* and *B. mallei* O-polysaccharides expanded between the region of 15–25 ppm. (A) DW503, (B) BT604 and (C) ATCC 23344. The peaks around 16 ppm represent 6-deoxyhexose CH_3 signals while those around 21 ppm represent *O*-acetyl (CH_3CO) signals.

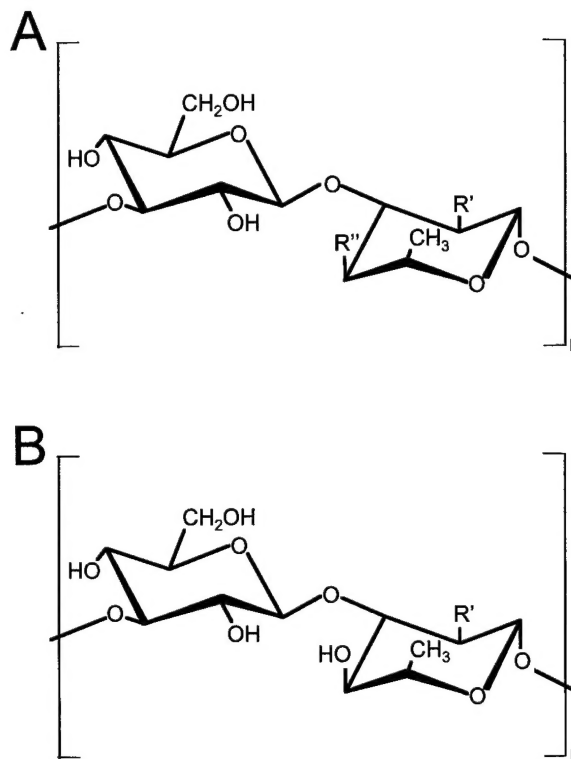


Fig. 5. Structures of (A) *B. pseudomallei* O-PS II and (B) *B. mallei* O-PS. In *B. pseudomallei* $\text{R}' = \text{O-methyl or O-acetyl}$ and $\text{R}'' = \text{O-acetyl or OH}$. In *B. mallei* $\text{R}' = \text{O-methyl or O-acetyl}$.

tra consistent with that obtained for *B. mallei* O-PS (Fig. 4). Similar conclusions can also be drawn for *B. pseudomallei* PB604. Based upon these observations, it is highly probable that a second unlinked locus is responsible for the *O*-acetylation of L-6dTalp residues at the *O*-4 position since the *wbiA* locus is the only predicted *trans*-acylase in the O-PS II biosynthetic operon. Studies are currently under way to examine this hypothesis.

3.4. Characterization of the epitope recognized by the Pp-PS-W mAb

We have recently demonstrated that the O-PS antigen expressed by *B. mallei* does not react with Pp-PS-W [11]. A comparison of the O-antigens expressed by *B. pseudomallei* and *B. thailandensis* with those expressed by *B. mallei* strains suggested that this phenomenon was likely due to differences in the *O*-acetylation patterns exhibited by the O-PS and O-PS II molecules (Fig. 5). Based upon the results of the current study, it is now apparent that the mAb reacts only with 3)- β -D-glucopyranose-(1 \rightarrow 3)-6-deoxy- α -L-talopyranose-(1 \rightarrow polymers in which the L-6dTalp residues are coordinately acetylated at the *O*-2 and *O*-4 positions. Whether or not the 2-*O*-acetyl modification imposes conformational constraints upon the O-polysaccharides or serves more directly as a structural epitope remains yet to be determined. Needless to say, however, these observations have proven to be a valuable re-

minder of the importance of maintaining the structural integrity of O-PS II during the synthesis of the glycoconjugate vaccine candidates.

Acknowledgements

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